THE ROLE OF THE NOTCH-3 RECEPTOR IN STEMNESS, EMT, AND TUMORIGENESIS IN COLORECTAL CANCER CELLS

By

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As the second leading cause of cancer death worldwide, colorectal cancer (CRC) poses a significant threat to both men and women alike. Although a small percentage of CRC cases are inherited, the majority of cases arise from environmental causes and are widely attributed to dysfunctional cellular pathways. Currently, the CRC progression model maintains that cancer growth, relapse and metastasis are due primarily to cancer stem cells (CSCs), a cell subpopulation that shares many properties with stem cells through epithelial to mesenchymal transition (EMT). We have recently reported that Notch-1 signaling is highly associated with promoting cancer stemness and EMT in CRC. Furthermore, we observed that expression of the Notch-1 receptor also upregulates Jagged-1, a ligand of the Notch receptors, which may affect stemness and EMT. However, this effect is reversed by treatment with DAPT, a γ-secretase and Notch signaling inhibitor. Thus, we hypothesized that the effect of Notch-1 on stemness and EMT in CRC is mediated by Jagged-1 via another member of the Notch pathway, the Notch-3 receptor.

To assess our hypothesis, we utilized the colon cancer cell line HCT-116. The parental HCT-116 cells were transduced with an IRES (internal ribosome entry site)-GFP retrovirus that expressed the human intracytoplasmic domain of Notch-1, thus producing the ICN1 cell line. The ICN1 cells were then transduced with a small hairpin RNA (shRNA) construct that effectively knocked down Notch-3, which created the ICN1-shN3 cell line. Growth ability, plating efficiency and colosphere

formation were assessed in each cell line. In these experiments, cell culture and Western blot analysis were performed using standard methodology.

We found that targeting Notch-3 via shRNA transduction in the colon tumor cell line ICN1-shN3 resulted in a significant decrease of Notch-3 expression. Further Western blot analysis indicated reduced expression of CD44 and Slug, markers for stemness and EMT, respectively. Further analyses showed that in the absence of functioning Notch-3, plating efficiency decreased by 60% and migration decreased by 22% when compared to the ICN1 cell line. These results were accompanied by significant changes in colosphere formation when the ICN1-shN3 cells were compared to ICN1 cells. ICN1-shN3 cells showed a 35% reduction in colosphere formation compared to the ICN cells. This difference was observed in colospheres of both high (33%) and medium/low (37%) proliferative capacity.

These data indicate a key role for Notch-3 signaling in Notch-1-induced tumorigenesis mediated by Jagged-1. They also highlight the potential use of Notch-3 inhibitors to effectively target aggressive CRC tumors.

THE ROLE OF THE NOTCH-3 RECEPTOR IN STEMNESS, TUMORIGENESIS, AND EPITHELIAL TO MESENCHYMAL TRANSITION IN COLORECTAL CANCER CELLS

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DISCLAIMER

Part of this thesis contains data that has been published in the Journal of Cellular Biochemistry, titled “Notch-1 promotes Stemness and Epithelial to Mesenchymal Transition in Colorectal Cancer” (Fender et al., 2015).

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ADAM	A disintegrin and metalloproteinase
APC	Adenomatous polyposis coli
BCA	Bicinchoninic acid
BMP	Bone morphogenic protein
BMPR	Bone morphogenic protein receptor
BSA	Bovine serum albumin
CBF1	C-promoter binding factor
CDK	Cyclin dependent kinase
Co-R	Co-repressors
CRC	Colorectal cancer
CSC	Cancer stem cell
CSL	Cbf1, suppressor of Hairless, Lag-1
DAPT	N-[(3,5-Difluorophenyl)acetyl]-L-alanyl-2-phenyl]glycine-1,1-dimethylethyl ester
DLL-1, -3, -4	Delta-like-ligand-1, -3, -4
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSL	Delta/Serrate/Lag-2
EDTA	Ethylenediaminetetracetic acid
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
Fbw7	F-box/WD (Tryptophan-Aspartic Acid) repeat domain-containing 7
GFP	Green fluorescent protein
GSK	Glycogen synthase kinase
HAT	Histone acetylase complex
HCl	Hydrochloric acid
HH	Hedgehog signaling pathway
ICN	Intracellular domain of Notch-1
ISC	Intestinal stem cells
IRES	Internal ribosome entry site
LNR	Lin12-Notch-repeat
LRP	Lipoprotein receptor-related protein
MAML	Mastermind-like proteins
MET	Mesenchymal-to-epithelial transition
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
shRNA	Small hairpin ribonucleic acid
NEXT	Notch extracellular truncation
NLS	Nuclear localization signal
O-fut	O-fucosyl-transferase

PEST	Peptide sequence of proline, glutamic acid, serine, threonine
PVDF	Polyvinylidene fluoride
RAM	Rbp associated molecule
RPM	Rotations per minute
RTK I, II	Receptor tyrosine kinase I, II
S1, S2, S3	Cleavage site 1, 2, 3
SIS3	Specific inhibitor of Smad-3
TAD	Transactivation domain
T-ALL	T-cell acute lymphoblastic leukemia
TBS	Tris-buffered saline
TBST	Tris-buffered saline + tween 20
TCF/LCF	Transcription coactivator of transcription factors/lymphoid-enhancer binding factor
TGF-β	Transforming growth factor- beta
TSG	Tumor suppressor genes
α	Alpha
β	Beta
γ	Gamma

INTRODUCTION

CRC Incidence, Risk Factors, and Treatments

Each year, roughly 1.2 million people are diagnosed with colorectal cancer (CRC) worldwide (American Cancer Society (ACS), 2014). Of these patients, over 600,000 will die from the disease (ACS, 2014). Since the mid-1980s, CRC incidence has steadily decreased (ACS, 2014). Among those diagnosed with CRC, the overall survival rate has increased, reaching as high as 65% in some developed countries and 50% in undeveloped countries (Brenner et al., 2014). The declines in CRC incidence and mortality over the past decade are largely due to frequent sigmoidoscopy and colonoscopy screening procedures, which permit detection and removal of precancerous polyps (Vinson et al., 2015). If left untreated, non-cancerous polyps can develop into cancerous lesions (Brenner et al., 2014).

There is a broad spectrum of risk factors that lead to the development of CRC, including sex, age, lifestyle, diet, and medical and family history (Vinson et al., 2015). The disease affects 30-40% more men than women (ACS, 2014). In 90% of patients, the onset of CRC occurs after the age of 50, and the median age at the time of diagnosis is 70 years (ACS, 2014). The risk of developing CRC is greater in those individuals who have conditions such as inflammatory bowel disease, diabetes, or obesity (ACS, 2014). Risk of CRC is also elevated in those who smoke, consume alcohol excessively, are physically inactive, or eat large amounts of red or processed meat (Vinson et al., 2015). Although up to 35% of CRC cases may have a heritable component, only a small fraction (<5% of cases) can be attributed to a well-defined, inherited genetic mutation (Brenner et al., 2014). Thus, for the vast majority of patients with inherited CRC, the specific mechanisms of inheritance are unclear (Brenner et al., 2014).

Fortunately, modern medicine and lifestyle changes have been shown to reduce one's risk of CRC by as much as 30% (Brenner et al., 2014). These beneficial lifestyle changes include increased physical activity, taking aspirin regularly, hormone replacement therapy, and regular screening for precancerous lesions (Brenner et al., 2014). Following a diet that is rich in fruit, vegetables, fiber, whole grains, dairy products, fish, and Vitamin D may also offer a slight protective effect (Brenner et al., 2014).

Cancerous lesions in the large intestine develop over a period of roughly 10 years (ACS, 2014). Lesions begin as non-cancerous polyps on the inner lining of the intestine and most commonly arise from the adenomatous tissue 96% of the time (ACS, 2014). Although fewer than 10% of non-cancerous polyps will develop into cancerous lesions, the likelihood that a polyp will become cancerous increases as it grows larger (ACS, 2014).

Current treatments for CRC include combination chemotherapy, surgery, radiation, and some targeted therapies (Vinson et al., 2015). Because CRC arises from the dysregulation of certain signaling pathways, identifying these pathways and their specific mechanisms is the key to creating more effective treatments for CRC patients (Vinson et al., 2015). Once cellular pathways are further understood, small molecule inhibitors that function by directly targeting aberrant signaling pathways can prevent cancer progression. Such inhibitors, in combination with chemotherapy and surgery, can provide better outcomes for patients with CRC. (Vinson et al., 2015).

In the field of oncology, tumorigenesis has been a significant topic of study for many decades. In 1985, a study that collected data from various tissue samples proposed that most colorectal carcinomas arise from adenomas, which increase in size, dysplasia, and degree of villous morphology over a period of 10 years (Sugarbaker et al., 1985). Five years later, a

different group developed the Vogelstein model, which identifies four distinct components of CRC tumorigenesis. The first of these proposed that colorectal tumors develop as a result of mutational activation of oncogenes and pronounced increases in mutational deactivation of tumor suppressor genes (TSGs). Second, malignant tumor formation requires more than one mutation at a single gene. Third, the summation of the mutations (Ras oncogene mutations and chromosomal deletions), not the order of mutations, determine the tumor's biological fate. Finally, not all of the TSGs will act in an autosomal recessive manner (Vogelstein & Fearon, 1990).

Epithelial Development of the Colon

In the inner lining of the large intestine (also known as the intestinal mucosa), the basic unit of structure is the crypt *(Fig. 1)*. The base of each crypt is composed of undifferentiated intestinal stem cells (ISCs) and progenitor cells, both of which are crucial for self-renewal. Formation of differentiated daughter cells compose the epithelial lining of the intestine. As the cells divide and grow from the lower stem-cell region, they move up the crypt into the proliferative zone, where they are then classified as transit amplifying cells. Because they divide twice daily, transit-amplifying cells are responsible for the rapid replacement of the intestinal epithelium. Further differentiation occurs as the transit-amplifying cells ascend to the upper-most portion of the crypt known as the differentiative zone, where they ultimately differentiate into goblet cells, enterocytes and neuroendocrine cells. When cells reach the lumen of the large intestine, they are eventually shed and undergo apoptosis. This continuous process is controlled by the crypt's stem cells and gradients formed from various signaling pathways (Vinson et al., 2015).

Figure 1. *Colon Anatomy and Signaling in the Crypt of Liberkuhn*

The figure shows the overall structure and components of the human colon crypt. The presence of signaling pathways WNT, Notch, HH, and BMP is shown. The differentiative, proliferative and stem-cell zones, and the direction of stem cell differentiation are also indicated.

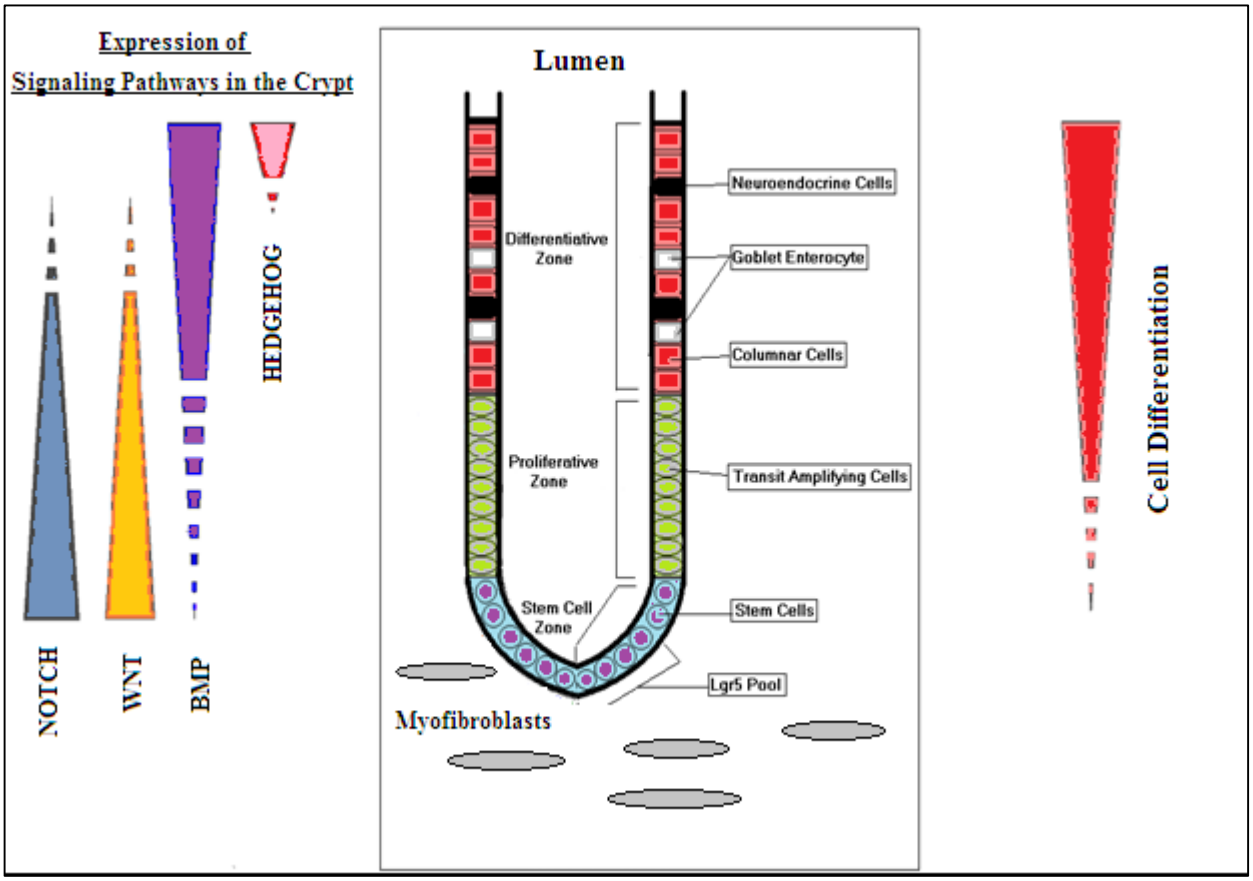


Figure 1. *Colon Anatomy and Signaling in the Crypt of Liberkuhn*

The cycle of continuous epithelial replacement is tightly controlled by a gradient of signaling pathways present in the crypt, which include WNT, Notch, BMP, and Hedgehog (HH) (Vinson et al., 2015). Of these, Notch signaling pathway is most notable in the stem cell compartment at the base of the colon crypt. In this region, proper Notch signaling is imperative to the proper differentiation, proliferation, and eventual apoptosis of epithelial cells (Previs et al., 2015). When dysregulated, the Notch pathway has been implicated in many cancers, including CRC (Medema and Vermeulen, 2011).

The Notch Pathway

In the human body, the Notch signaling pathway *(Fig. 2)* is composed of five ligands and four receptors (Vinson et al., 2015). The ligands, named Jagged-1, Jagged-2, Delta-like-1 (Dll-1), Delta-like-3 (Dll-3), and Delta-like-4 (Dll-4), are single-pass transmembrane proteins that contain epidermal growth factor (EGF)-like repeats (Vinson et al., 2015). The four receptors of the pathway, named Notch-1, Notch-2, Notch-3 and Notch-4, are initially synthesized as protein precursors, or "pre-Notch" receptors (Al-Hussaini et al., 2011). As development of the receptor continues, pre-Notch receptor proteins are fucosylated by the chaperone O-fucosyltransferase (O-Fut) in the endoplasmic reticulum *(Fig. 2a)* (Vinson et al., 2015). It then enters the golgi apparatus, where Fringe glycosyltransferases (Lunatic, Manic and Radical) extend the O-fucose moieties and modify the receptor for interaction with a specific ligand *(Fig. 2b)* (Vinson et al., 2015). The receptor remains in the golgi while a proteolytic cleavage by a Furin-like protease at site 1 (S1) occurs, which heterodimerizes the Notch receptor *(Fig. 2c)* (Vinson et al., 2015). In the last stage of synthesis, the mature Notch receptor is targeted to the cell membrane where it is held in place by non-covalent interactions (Vinson et al., 2015).

Figure 2 (a-f). *The Notch Signaling Pathway and its Effect on the Activation of Target Genes*
Pre-Notch first undergoes fucosylation by O-fut (O-fucosyl transferase) in the ER *(A)*, then is glycosylated and cleaved (S1 cleavage) by a furin convertase in the *trans*-golgi *(B)*. The heterodimeric receptor is assembled on the cell surface, where it is available for ligand binding *(C)*. Upon ligand binding, Notch undergoes S2 cleavage on the outer plasma membrane by ADAM (a disintegrin and metalloproteinase) and S3 cleavage at the inner plasma membrane by gamma-secretase, and then is transported to the nucleus where it activates transcription of DNA *(D)*. CoR (co-repressors) are displaced by ICN and transcription proceeds by CSL (CBF1, suppressor of hairless, Lag-1), MAML (mastermind-like proteins), HAT (Histone acetylase complex) and p300 *(E)*. Notch signaling is terminated when the ICN is targeted for degradation *via* the ubiquitin pathway *(F)*.

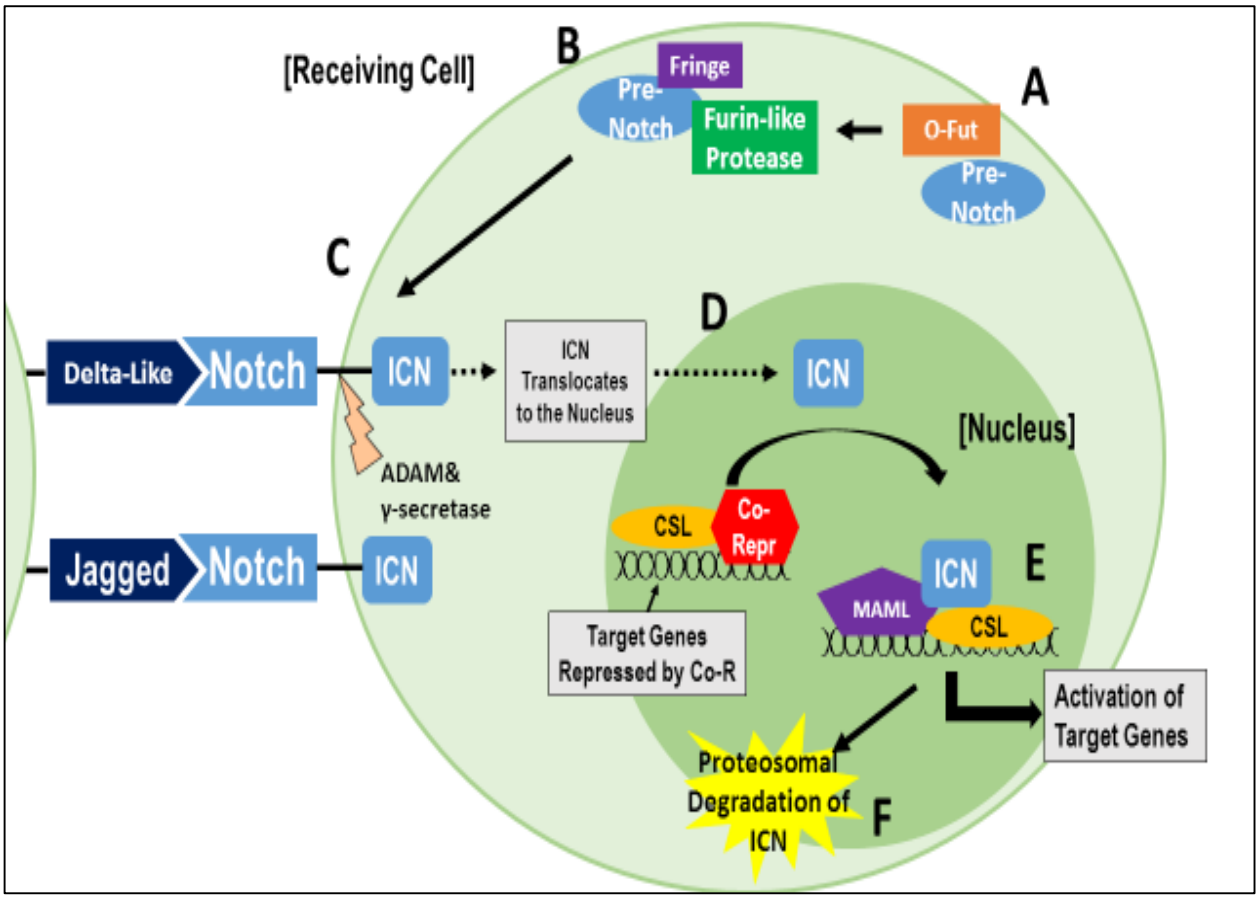


Figure 2(a-f). *The Notch Signaling Pathway and its Effect on the Activation of Target Genes.*

The Notch receptors are transmembrane proteins with two distinct domains: extracellular and intracellular *(Fig. 3)* (Vinson et al., 2015). The extracellular domain contains variable numbers of EGF-like repeats, which permit binding affinity of particular ligands (Vinson et al., 2015). Notch receptors differ in their number of extracellular EGF-like repeats; Notch-1 and Notch-2 contain 36 repeats, Notch-3 contains 34 repeats, and Notch-4 contains 29 repeats (Ntziacristos et al., 2014). All Notch extracellular domains contain three LNR (LIN-12/Notch-related) residues that prevent ligand-independent signaling, as well as two cysteine residues (Al-Hussaini et al., 2011). The intracellular domain of the Notch receptor (ICN) functions as a transcriptional activator, and consists of ankyrin repeats, a RAM (Rbp-associated molecule) domain, a TAD (transactivation domain), an NLS (nuclear localization signal), and a PEST (proline-, glutamate-, serine-, and threonine-rich) domain that is responsible for the stability of proteins and the facilitation of rapid proteolytic degradation (Bertrand et al., 2012). Although the four Notch receptors differ slightly in structure and possess specific ligand affinities, they operate using the same basic signaling pathway (Al-Hussaini et al., 2011).

Notch signaling is activated when a Notch ligand binds to its Notch receptor. This begins a sequence of two proteolytic cleavages of the Notch receptor *(Fig. 2)* (Vinson et al., 2015). Notch is first cleaved by ADAM (A disintegrin and metalloprotease) 10/17 directly outside the cell membrane at 'site 2', which removes Notch's extracellular domain (Vinson et al., 2015). The remaining intracellular portion of the Notch receptor is referred to as the NEXT (Notch extracellular truncation) domain.

Figure 3: *The Notch Family Receptors.*

Notch-1, -2, -3, and -4 receptors shown with their various components. EGF: epidermal growth factor; LNR: Lin Notch repeat; RAM: Rbp-associated molecule; PEST: peptide sequence of proline, glutamic acid, serine and threonine.

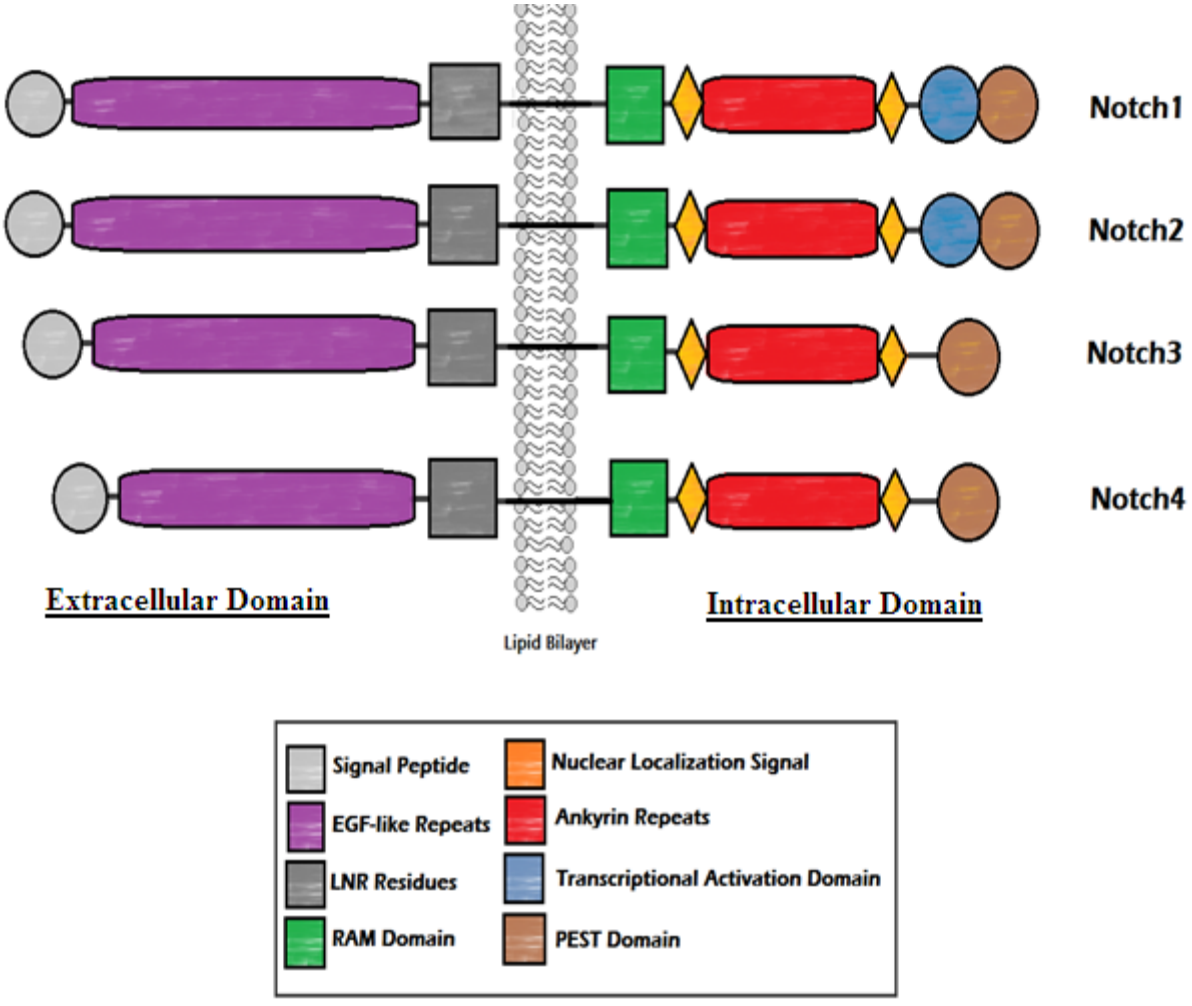


Figure 3. *The Notch Family Receptors*

The NEXT domain is subsequently cleaved at the inner cell membrane, 'site 3' (S3), freeing the ICN, which then translocates to the nucleus *(Fig. 2d)* (Vinson et al., 2015). In the nucleus, ICN engages with DNA-binding proteins to regulate gene expression and initiate transcriptional events (Bertrand et al., 2012). ICN first binds to an inactive CSL (CBF-1/Suppressor of hairless/Lag-1) transcription factor (Vinson et al., 2015). Coactivators, including mastermind-like proteins, p300, and the histone acetyl transferase complex (HAT), displace the co-repressors (Co-R) that are bound to CSL, which transforms the ICN-CSL complex into a transcriptional activator (Karamboulas and Ailles, 2013). The downstream targets of the transcription include cell-fate regulators of the HES and HEY families, which are composed of a basic domain (determining DNA-binding specificity), and a helix–loop–helix domain (allowing for dimer formation) (Aithal and Rajeswari, 2013). The dimerization of HES and/or HEY proteins regulates the transcription of key genes involved in apoptosis, cell cycle regulation, proliferation and differentiation, neurogenesis, and metabolism (Al-Hussaini et al., 2011).

A new cycle of Notch signaling begins after the synthesis of the pathway's targets has occurred (Vinson et al., 2014). At this time, ICN is phosphorylated by CDK8 (cyclin-dependent kinase 8) on its PEST domain *(Fig. 2e*) (Schwanbeck, 2015). The phosphorylation allows ubiquitination by the E3 ubiquitin ligase Fbw7 (F-box and WD repeat domain-containing 7), followed by proteosomal degradation (Vinson et al. 2015). Ultimately, the phosphorylation and ubiquitination of the ICN terminate Notch signaling, and the cell is reset to begin a new cycle. (Bertrand et al., 2012).

The Role of Notch Signaling in CRC

Dysregulation of the Notch pathway is commonly associated with many cancers. Aberrant Notch signaling was first identified in Leukemia, specifically T-cell Acute Lymphoblastic Leukemia (T-ALL) (Bertrand et al., 2012). In T-ALL, Notch-1 was found to be constitutively activated upon fusing to the T-cell receptor B-locus (Bertrand et al., 2012). Later research demonstrated that mutations in Notch-1 are highly prevalent among T-ALL patients, further implicating the oncogenic role of Notch-1 (Bertrand et al., 2012).

Depending on the specific microenvironment, the Notch receptors can act as both oncogenes and tumor suppressors. It is widely recognized that upregulated levels of Notch-1 and Notch-3 are associated with cancer progression, tumor metastasis and inhibition of apoptosis (Vinson et al., 2015). However, unlike Notch-1, the upregulation of Notch-2 is associated with better clinical outcomes (Vinson et al., 2015). Thus, it is clear that the diverse functions of the Notch receptors contribute in contradicting ways to the progression of CRC (Bertrand et al., 2012).

Cancer Stem Cells

The differentiating cells of the intestinal crypt are dependent upon the tight regulation of signals arising from the supportive mesenchymal cells and the extracellular matrix (ECM). The crypt's stem-cell compartment is predominantly regulated by Notch signaling, and deviations from its canonical pathway may contribute to the development of CSCs. In the colon, intestinal stem cells (ISCs) drive the regeneration of normal intestinal epithelium, and it is proposed that CRC shares this model of cell development and reproduction, where CSCs are responsible for tumor growth and proliferation (Bertrand et al., 2012). When Notch signaling in the crypt is dysregulated, an accumulation of mutations may result, promoting the development of CSCs,

and likely CRC. CSCs are defined by their ability to self-renew, expand and differentiate. Overall, it is imperative to further understand the signaling and regulation involved in CRC development and maintenance in order to improve the therapies and prognosis for CRC patients (Medema and Vermeulen, 2011).

Epithelial-to-Mesenchymal Transition

Recent studies have shown that metastasis of CRC is driven by Notch signaling through a process known as EMT (Zhao et al., 2015). During EMT, epithelial cells undergo a developmental switch, resulting in decreased adhesion, loss of cell polarity, increased proliferation, and motility. EMT is a normal process in some systems; the ability of various species to develop highly specialized tissues and organ systems is the result of increased differentiation of mesenchymal cells through EMT. Thus, many of the pathways involved in EMT are highly conserved and essential for development across different species (Lindsey and Langhans, 2014).

However, the benefits of EMT in organismal specialization have been challenged by EMT's role in the metastasis of certain carcinogenic tumors. Increased proliferation, motility, and invasion are key steps towards progression of a malignant phenotype, which occurs as tumor cells translocate from the initial lesion into neighboring tissues. Phenotypically, the cells change from a cuboidal, epithelial cell to a spindle-shaped mesenchymal cell (Lindsey and Langhans, 2014).

As EMT progresses, the cell surface changes dramatically. Generally, cells are dependent upon the extracellular matrix and cell-cell interactions for survival. When anchorage-dependent tumor cells become detached from the surrounding extracellular matrix, the signals

indicating cell survival cease, and a programmed cascade of events ends in cell death. Some cells that become detached from the ECM, thus losing the cell-survival signals, still manage to survive by evading the programmed death. This series of events leads to eventual metastasis (Heerboth et al., 2015).

In order to begin migration, the cells must alter their characteristics from epithelial to mesenchymal, down-regulate the receptors that aid in cell-cell attachment, and up-regulate cell adhesion molecules for movement. E-Cadherin, a transmembrane protein that anchors adjacent cells to each other forming adherens junctions, is lost. Snail, Zeb and Twist are known as E-Cadherin silencers/repressors. The loss of E-Cadherin and adherens junctions are pivotal steps in EMT and the key to metastatic migration of cells (Heerboth et al., 2015). This migration is facilitated by transcription factors Snail, Twist, Slug, Zeb, N-cadherin, and others. Successful metastasis involves not only the ability of a cell to migrate from their tissue of origin; the cells must then regain their epithelial characteristics in order to take root and begin differentiation in the secondary tissue *(Fig. 4)* (Heerboth et al., 2015).

Transcription factors Slug, Snail and TGF-β interact with the Notch pathway in a way that is critical for the process of EMT. Notch is of particular interest in CRC research because its signaling is prominent in the stem cell compartment of the colon crypt and has extensive relation to EMT transcription factors (Vinson et al., 2015).

Previous Work

It has recently been reported that Notch-1 signaling is highly associated with the promotion of cancer, stemness and EMT in CRC. Previous research indicates that constitutively activating the intracytoplasmic domain of the Notch-1 receptor in CRC cells (ICN1 cells)

resulted in the increased expression of Jagged-1, a ligand of the Notch pathway (Fender et al., 2015). In turn, the activation of Jagged-1 increases the expression of CD44 and Slug (Fender et al., 2015). This model is shown in *Figure 5*.

Since induction of stemness and EMT induced by Jagged-1 in parental CRC cells was blocked by DAPT, we assessed the expression of the Notch-2, -3, and -4 receptors in the presence of the ligand. We found that the Notch-3 receptor was highly upregulated when HCT-116 cells were cultured in plates coated with purified Jagged-1 *(Fig. 6)*. However, expression of Notch-2 and Notch-4 was not altered by the Jagged-1 ligand (data not shown).

Figure 4. *The Role of CSCs in Metastatic Colonization.*

The CSC model is a proposed theory for carcinogenesis. Formation of a primary tumor occurs and eventually begins to invade surrounding tissues. CSCs undergo the process of EMT and enter the circulatory system via intravasation. Eventually, CSCs leave the bloodstream via extravasation and migrate to distant tissues. In the final stage of metastasis, CSCs undergo mesenchymal to epithelial transition (MET) and tumor formation occurs.

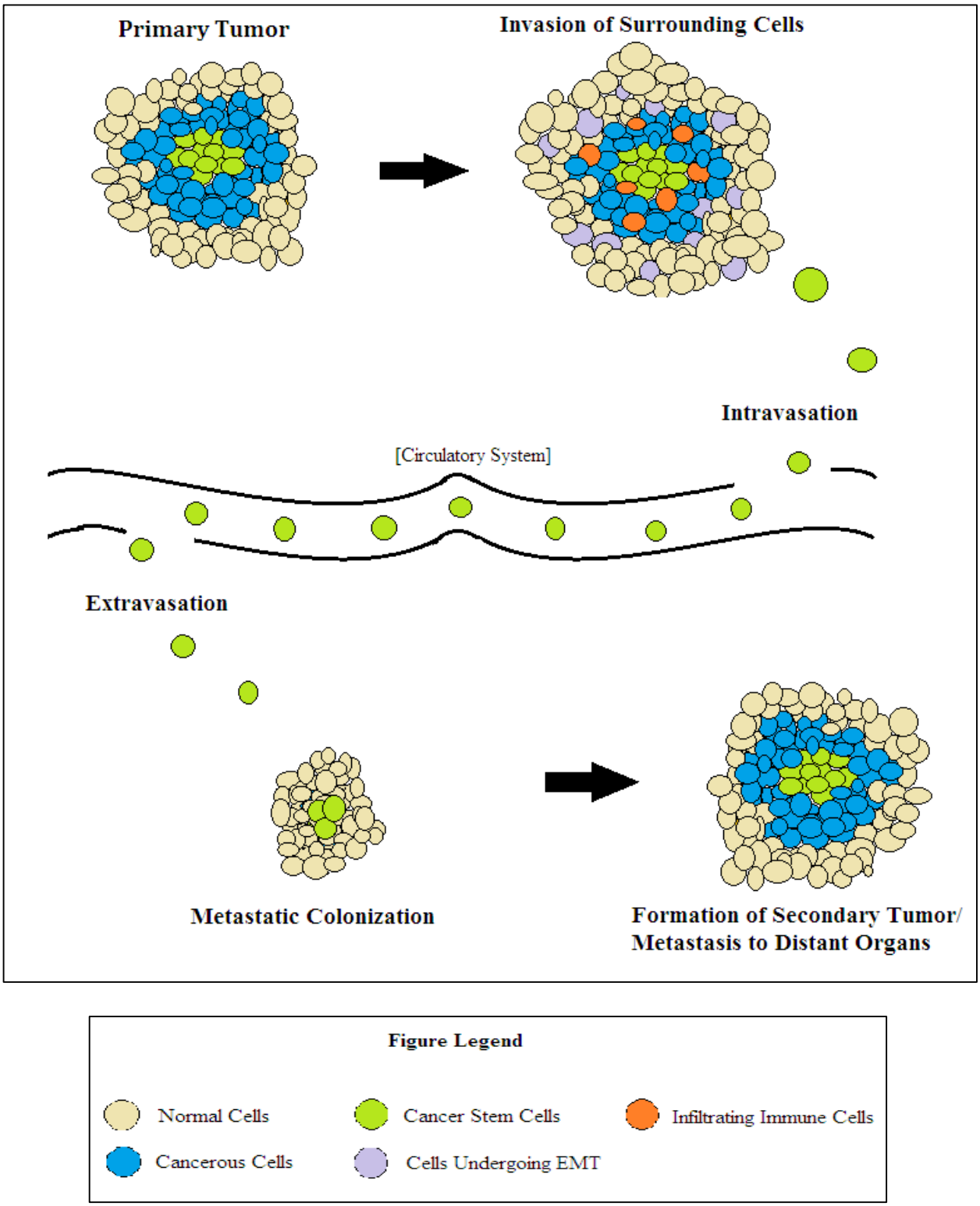


Figure 4. *The Role of CSCs in Metastatic Colonization*

Figure 5. *Previously Proposed Model.*

Notch-1 signal upregulates Jagged-1, which in turn activates another Notch-receptor species (that is DAPT sensitive). This ultimately leads to increased expression of CD44 and Slug, and thus, EMT (3).

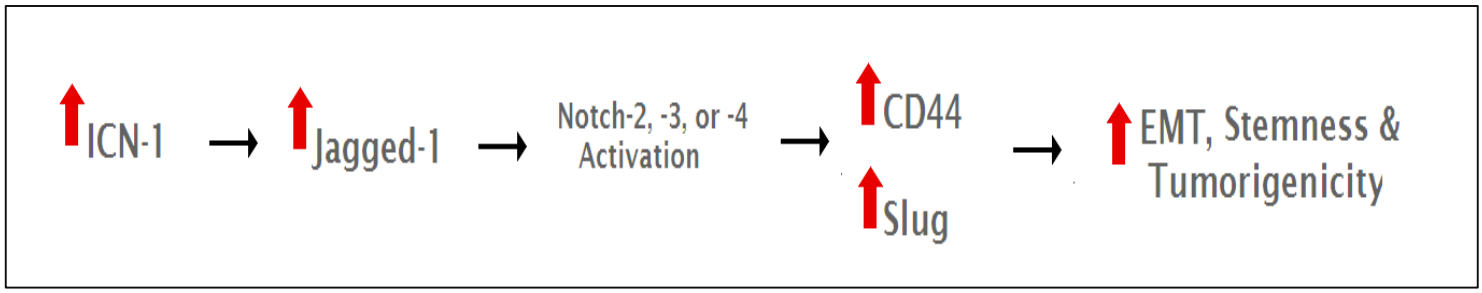


Figure 5. *Previously Proposed Model.*

Figure 6. *Jagged-1 Upregulates Expression of the Notch-3 Receptor in CRC cells.*

Proteins from whole cell lysate were analyzed with Western blot analysis from the HCT-116 cell line cultured in the presence of Jagged-1 and/or DAPT with the indicated antibodies. [(+), with; (-) without]

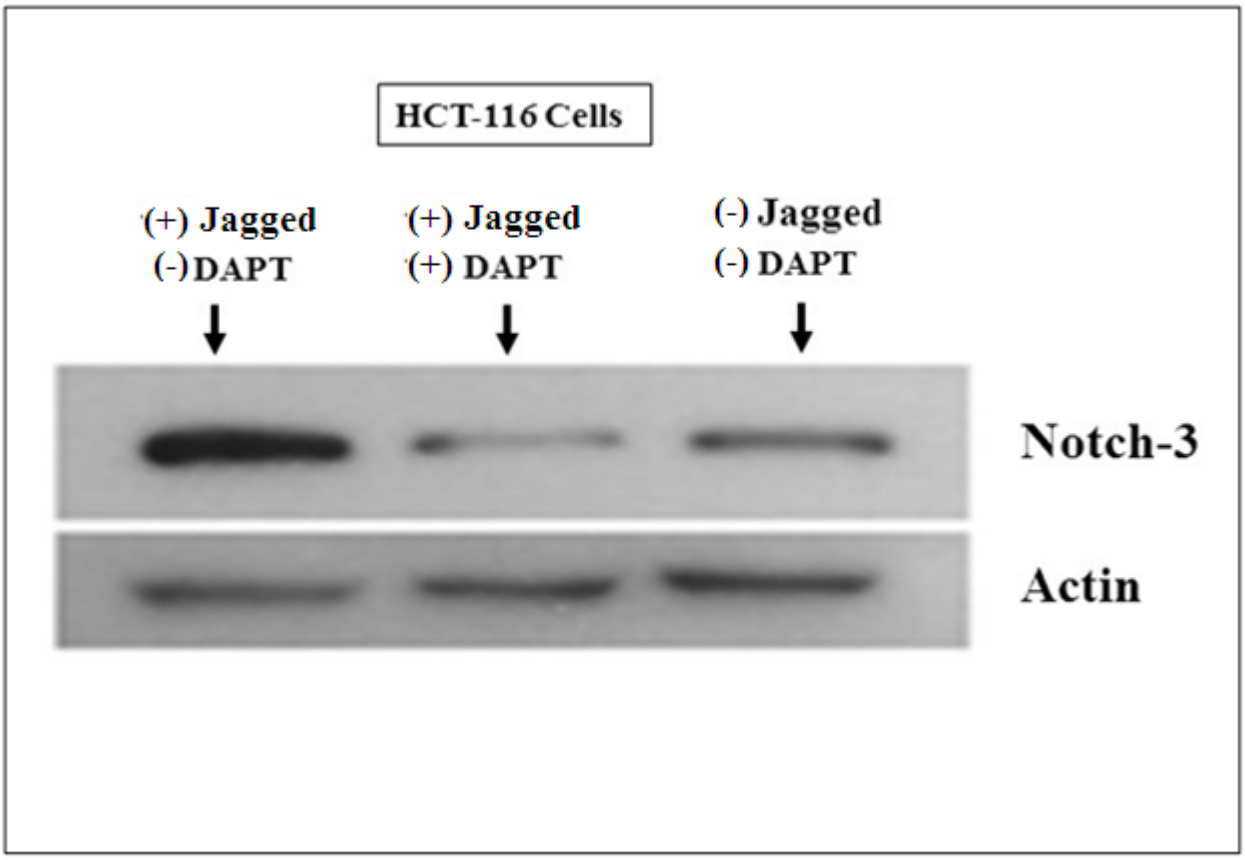


Figure 6. *Jagged-1 Upregulates Expression of the Notch-3 Receptor in CRC cells.*

HYPOTHESIS

Based on our previous findings, we hypothesized that Notch-1 receptor signaling promotes EMT, stemness, and tumorigenesis via activation of the Notch-3 receptor in colorectal cancer cells.

MATERIALS AND METHODS

Cell Lines and Culture

All cells were plated and grown in T-25 culture flasks and incubated at 37.0^{o}C with 4.0% CO_2. The flasks contained McCoy's Media (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) and 1% Penicillin-Streptomycin (Life Technologies). When confluent, the cells were passaged using 1xTrypsin/EDTA (ethylenediamenetetraacetic acid) (Life Technologies), and washed using Dulbecco's phosphate-buffered saline (Life Technologies).

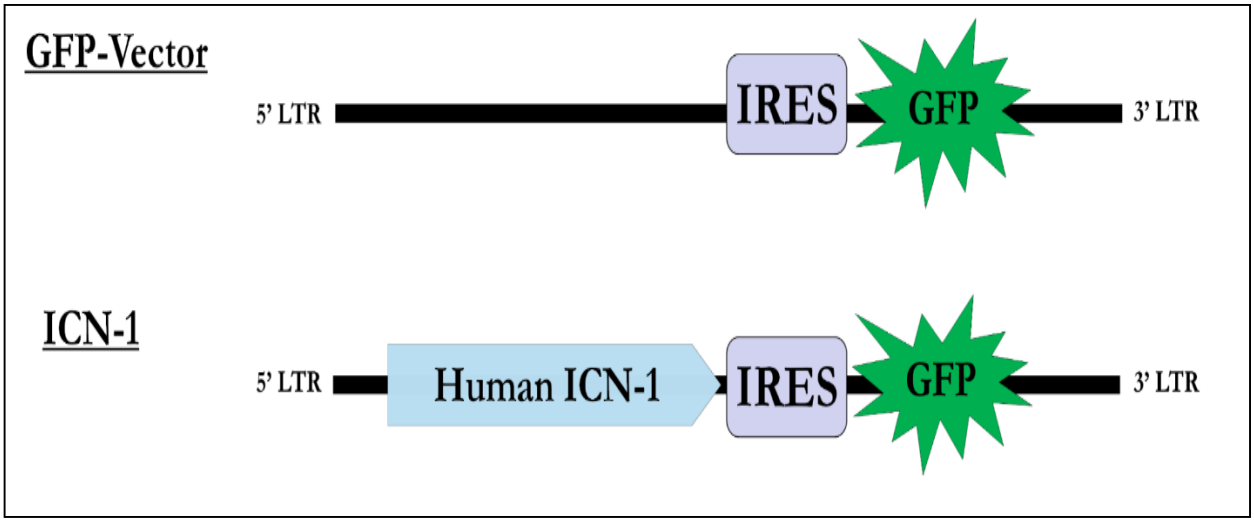
Multiple cell lines were developed from the parental cell line, HCT-116 (ATCC, Manassas, VA), which are derived from human epithelial colorectal carcinoma cells of an adult male. This cell line containing a functional Notch pathway is capable of tumorigenesis, and can also function as a transfection host.

A cell line that constitutively expressed the intracytoplasmic domain of the Notch-1 receptor (ICN1) was derived from the parental HCT-116 cells. The ICN1 cell line was generated by transducing HCT-116 cells with an IRES-GFP retrovirus that contained the human ICN1 (*Fig. 7a)*. The retrovirus was packaged by co-transfection with a plasmid that expressed the amphotropic viral coat. The viral particle of interest, packaged in the supernatant, was collected and plated over HCT-116 cells. Following this transfection, FACS (fluorescence-activated cell sorting) was used to collect those cells that simultaneously expressed the GFP-vector and the constitutively active ICN1. HCT-116 cells were also transduced with the empty IRES-GFP vector. Cell sorting was used to identify those cells expressing the GFP-vector alone (GFP-V cells), which were then collected and used as control 1 (*Fig. 7b)*.

Figure 7. *Creation of the Experimental CRC Cell Lines.*

HCT-116 cells were transduced with an IRES-GFP retroviral construct expressing the human intracytoplasmic domain of Notch-1 (ICN1) and the green fluorescent protein (GFP) gene *(A)*. The ICN1 cells were transduced with a small hairpin RNA construct that effectively knocked down Notch-3 and created the ICN1-shN3 cell line. Notch-3 null cells were selected in the presence of Puromycin. Cells expressing GFP (R1 region) were separated from cells lacking GFP expression to enrich the HCT116-ICN1 (ICN1) and the HCT-116-ICN1-shNotch3 (ICN1-shN3) cell lines using the Becton Dickinson FACSVantage SE cell sorter *(B)*.

(A)



(B)

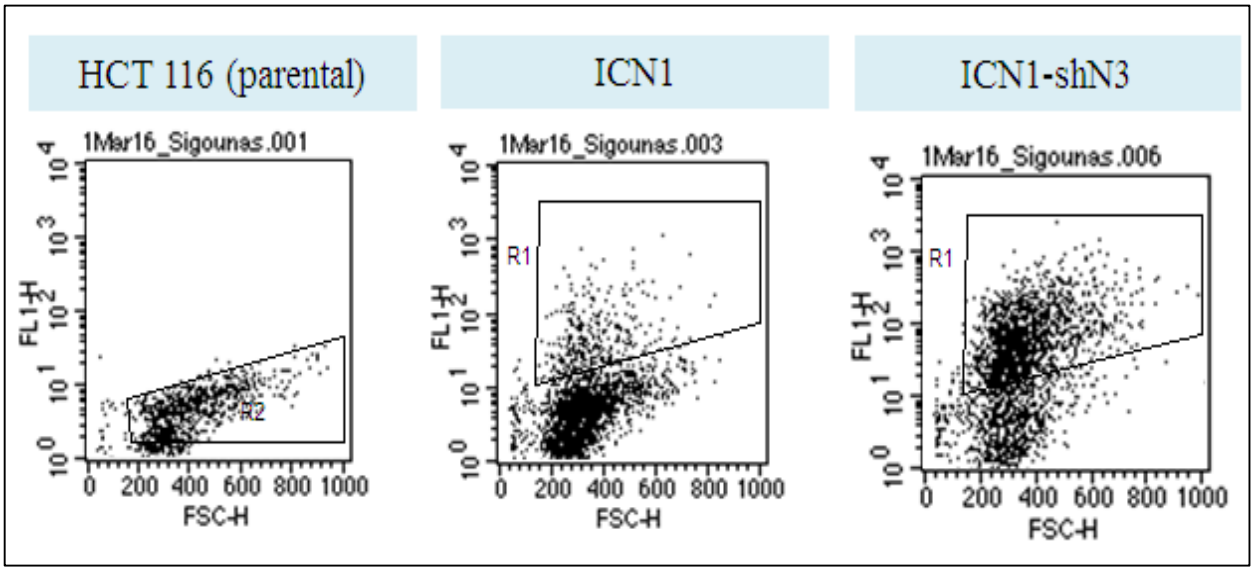


Figure 7. *Creation of the Experimental CRC Cell Lines*

In order to further identify the role of the Notch-3 receptor in the Notch signaling pathway, expression of the Notch-3 receptor was knocked down within the ICN1 cell line. Notch-3 shRNA lentiviral particles (Santa Cruz, Dallas, TX) were used to target the Notch-3 receptor for knockdown gene expression in the ICN1 cells as per manufacturer. For the transfection, 6µl shRNA duplex was diluted into 100 µl of transfection medium, and this solution was added to the shRNA transfection reagent provided. The ICN1 cells were incubated with the transfection reagent/medium solution for 24 hours. The cells' resistance to puromycin (1 µg/mL) was tested, and those cells surviving were further cultured. Because these new cells also expressed ICN1, the GFP-vector was selected by FACS. Specifically identifying the cells that were Puromycin resistant and positive for the GFP-vector further generated a more purified population of interest. These ICN1 cells with inhibited Notch-3 expression were given the name ICN1-shN3. HCT-116 cells that were transduced with scrambled shRNA and puromycin resistant were derived and used as control 2.

Purified Notch Ligand

HCT-116 cells were cultured in the presence of purified Notch ligands. Culture dishes were coated with purified Jagged-1 ligand (purchased from R&D systems) and subsequently overlaid with HCT-116 cells. Proteins were obtained from the lysates of these cells, and Western blot analysis was used to identify properties associated with stemness and EMT.

Pharmacologic Inhibition of Notch

The pharmacologic inhibition of Notch signaling using DAPT (*N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester*) has been widely studied as a

potential therapeutic approach to certain cancers. DAPT functions to inhibit the work of γ-secretase, which cleaves the Notch receptors from the cell membrane, thus directly affecting Notch signaling. However, because ICN1 represents the cleaved intracytoplasmic domain of the Notch-1 receptor, its expression is not dependent upon γ-secretase function. Thus, ICN1 expression is not affected by DAPT treatment.

Protein Isolation

All cells were grown in complete McCoy's 5A medium supplemented with 10% FBS and 1% Penicillin-Streptomycin. When protein was collected, the cells were released from the bottom of the flask by trypsinization, and immediately resuspended in media. Each cell suspension was centrifuged for 10 minutes at 1000 RPM. The media (supernatant) was then aspirated. A solution of lysis buffer, protease inhibitors (Sigma), and phosphate inhibitors 2 and 3 (Sigma) was used to break up the pellet containing whole cells by pipetting up and down several times. The cell samples were placed on ice for 30 minutes and vortexed for 30 seconds every 5 minutes. To separate the cell proteins from the rest of the cellular debris, the samples were centrifuged at 14,000 RPM for 15 minutes at 4^{o}C. Then, the supernatant was collected. Aliquots of each collected sample were stored at -80^{o}C until use.

BCA Assay

In order to determine the concentration of the proteins collected in the procedure mentioned previously, a BCA (bicinchoninic acid) assay was used. Serial dilutions of BSA (Bovine Serum Albumin) standard and dilutions of the unknown concentration protein collection sample were exposed to BCA working reagents (Sigma). Colorimetric analysis was performed

at 750nm using a plate reader. A standard curve was prepared based on the serial dilutions of the BSA standard. The concentration of each sample was then identified by comparing the sample absorbance at 750nm to the standard curve.

Western Blot Analysis

Whole cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and subsequently transferred to a PVDF (polyvinylidene fluoride) membrane. Each protein sample of 50 μg was combined with an equal volume of sample buffer (Biorad, Hercules, CA) with added reducing agent 2-Mercaptoethanol (Biorad). The samples were boiled in H_2O for 5 minutes and loaded into a pre-cast 10.5-14% 18-well Tris-HCl (hydrochloric acid) gel (Biorad). The protein sample mixtures, as well as a standard Kaleidoscope marker (Biorad) were loaded into the gel.

The gel was then transferred to a PVDF membrane (Biorad) overnight at 4^oC. Following the transfer, the membrane was blocked for 2 hours in a 5% nonfat milk solution in 0.1% TBST (Tris-Buffered Saline +Tween 20). Primary antibody incubation occurred overnight at 4^oC. In all experiments, actin protein was used as a loading standard. The following day, five consecutive 10 minute washes in TBST were used to wash the excess primary antibody from the membrane. Then, incubation with the secondary antibody for 2 hours at room temperature. Excess secondary antibody was removed by four consecutive 10 minute washes in TBST, followed by a single 10 minute wash in TBS.

A chemiluminescent substrate (Thermo Scientific, Rockford, IL) was added to the membrane and the Western blot was exposed onto an x-ray film. After developing the film, the

Q-scan software program (Biosoft, Cambridge, UK) was used to quantify the individual band density.

Duplication Time Assay

For each cell line, 2,000 cells were plated separately into 35 x 10 mm culture dishes containing 3 mL of complete media. After 7 days of growth, each culture was trypsinized to completely remove the cells adhered to the bottom of the dish. The cells were counted manually using a hematocytometer as well as using an automated cell counter (Life Technologies). The duplication time of each cell line was determined from these cell counts using the formula $d = [t\ (ln\ (2))] \div [ln\ (N_T/N_o)]$ where $\boldsymbol{d}$ = duplication time (in hours), $\boldsymbol{t}$ = hours of growth, $\boldsymbol{N_T}$=number of cells at the time of counting, and $\boldsymbol{N_o}$=number of cells originally plated.

Plating Efficiency

Using a FACSVantage SE cell sorter (Becton Dickinson, Franklin Lakes, NJ), a single cell along with complete culture media was plated into each well of a 96-well plate. Each of the cell lines HCT 116, ICN1, ICN1-shN3, and controls 1 and 2 were plated onto a separate 96-well plate. After 3 weeks of growth, the number of wells in each plate that contained colonies were counted. To assess cell viability/proliferation, we used a modified protocol from Sigma. Briefly, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide; thiazolyl blue) (Sigma) was dissolved in RPMI-1640 without phenol red (5 mg/ml final concentration). The solution was filtered through a 0.2 μm filter and stored at 2–8 °C for frequent use or frozen for extended periods. MTT stock solution (5 mg/ml) was added to each culture being assayed to equal one-tenth the original culture volume and incubated for two hours. At the end of the incubation period the converted dye was solubilized with acidic isopropanol (0.04-0.1 N HCl in absolute

isopropanol). Absorbance of converted dye was measured at a wavelength of 570 nm with background subtraction at 630–690 nm.

Wound Repair Assay

Cells were plated to confluency in culture plates. Using a 200 µl pipet tip, a single scratch was made in each well from the top to the bottom. Following the initial scratching, the width of each scratch was measured in two places using a microscope with an ocular ruler. After 24 hours, the scratches were measured again. The amount of cellular migration or 'wound repair' was determined by subtracting the second measurement from the first.

Anchorage Independent Growth assay

Equal parts of 1.6% liquid low melting point agarose solution and 2x RPMI media were mixed. 1.5 ml of this solution was dispensed into each well of a 12-well culture plate. This layer was then topped with a second layer of 1.5ml that contained equal parts of 0.8% agarose solution and 2x RPMI media. The second layer also contained 1,000 cells. For each cell line, three wells were filled.

The cells remained in culture at 37^{o}C for 10 days. On day 10, the cultures were removed from the incubator and stained with 125 µl of 2.4 mM MTT (MW 414.32 g). The MTT stain was visible in the culture colonies after 1 hour. The plates were stored long term at 4^{o}C.

Statistical Analysis

In order to evaluate differences between groups, variables were analyzed using the Student's t-test (Fender et al., 2014). The Student's t-test was two-sided and performed using Microsoft Excel. Statistically significant differences between groups were considered to have a

P-value of <0.05. The results are expressed as the mean ± standard error (SE) of at least three experiments.

RESULTS

Notch-3 Receptor Expression is Regulated by Jagged-1

Previous studies by Fender et al., 2015 have shown that increased expression of the Jagged-1 ligand induced by constitutively active Notch-1 was not greatly affected by DAPT (a Notch inhibitor) treatment. However, the increased expression of Slug and CD44 markers in ICN1 cells was significantly reduced following the DAPT treatment. To further explore these findings, our group cultured HCT-116 cells in the presence of a purified Notch ligand, Jagged-1. Cells were split into three treatment groups: (1) in the presence of the Jagged-1 ligand alone (2) in the presence of the Jagged-1 ligand and DAPT treatment, and (3) neither Jagged-1 ligand nor DAPT treatment.

We found that when the HCT-116 cells were exposed to Jagged-1 ligand alone (1), the Notch-3 expression increased by 30% (*Fig. 6*). However, when the Jagged-1 ligand and DAPT treatments were combined, Notch-3 expression was decreased by 63% *(Fig. 6)*. Further studies ruled out Notch-2 or Notch-4 as playing significant role (data not shown). Thus, these results turned our attention to the Notch-3 receptor as a possible mediator of stemness and EMT in Notch signaling, which is the underlying concept of this project.

Generation of the ICN1 and ICN1-shN3 Cell Lines

In order to further identify the function of the Notch-3 receptor within the previously proposed mechanism (*Fig. 5),* we used the ICN1 and ICN1-shN3 cell lines. Methodology regarding the derivation of ICN1 and ICN1-shN3 cell lines is noted in *Methods.* Western blot analysis was then performed to ensure the transfection with shRNA for Notch-3 was successful in knocking down the Notch-3 receptor in the ICN1 cells (*Fig. 8*).

Figure 8. *Detection of Notch-3 Receptor in ICN1-shN3 Cells.*

A Western blot confirms the presence of Notch-3 within the parental HCT-116, controls, and ICN1 cell lines. As expected, the lack of Notch-3 expression is apparent in the ICN1- shN3 cell line.

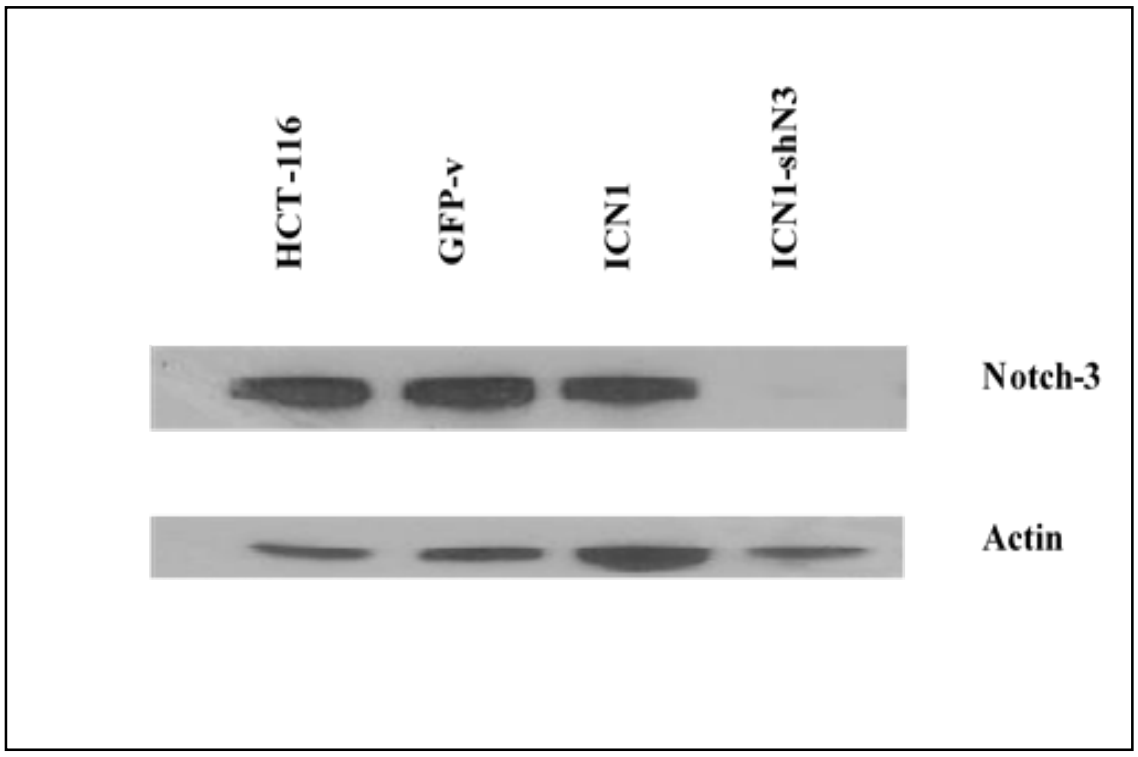


Figure 8. *Detection of Notch-3 Receptor in ICN1-shN3 Cells.*

Morphological Properties of the ICN1 and ICN1-shN3 Cell Lines

Using the EVOS microscope, images were captured from the HCT-116, ICN1 and ICN1-shN3 cell lines (*Fig. 9).* Morphological distinctions between each of the cell lines are shown. The HCT 116 cells are flat and epithelial-like. Compared to the HCT 116 cells, the ICN1 cells are rounder and more spindle-like, indicating a mesenchymal phenotype. The ICN1-shN3 cellular morphology suggests a phenotype sharing characteristics with both the HCT 116 and ICN1 cell lines. Expression of the GFP was detected in the ICN1 and ICN1-shN3 cells, but was absent from the HCT-116 cells.

Expression of CD44 and Slug Markers in ICN1-shN3 Cells

Western blot analysis was used to determine the expression of proteins associated with stemness and EMT *(Fig. 10)*. It is evident that there is expression of both CD44 and Slug in the ICN1 cells as opposed to the HCT-116 and other control lines. However, when Notch-3 is targeted in the ICN1 cells, the CD44 and Slug expression was eliminated.

Colony Size and Plating Efficiency of the ICN1-shN3 Cells

The colonies present were derived from a single cell as explained in *Methods*. As shown in *Figure 11*, individually plated ICN-shN3 cells formed the fewest number of overall colonies compared to ICN1 and control cells. In addition to counting the total number of colonies formed within each cell line, colony sizes were also determined. To determine colony size, colony dimensions were measured using a microscope with an ocular ruler. Furthermore, the colony size was determined by measuring the optic density in an ELISA reader using the MTT assay. Colonies were grouped into three categories based on their optic density [small (<0.1), medium (0.1-0.5) and large (>0.50)].

Figure 9. *Morphology of Experimental CRC Cell Lines.*

ICN1-transduced cells and ICN1 cells transduced with Notch-3 shRNA expressing GFP (R1 region of Figure 2B) were separated from cells negative for GFP expression to form the working ICN1 and ICN1-shN3 lines. Images of HCT-116, ICN1 and ICN1-shN3 cells were taken with the EVOS microscope in white (upper panels) and green fluorescent (lower panels) light at 20X magnification

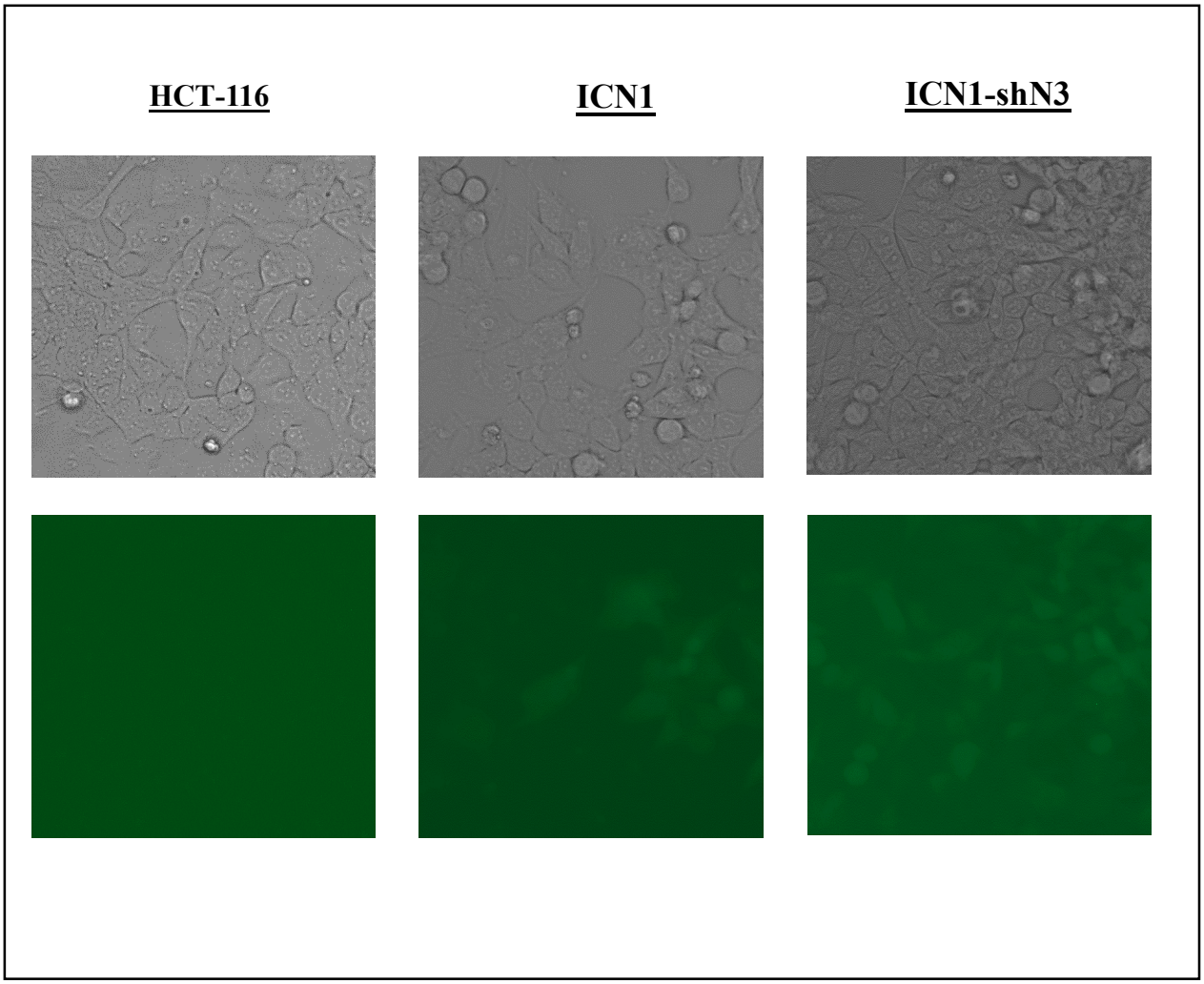


Figure 9. *Morphology of the Experimental CRC Cell Lines*

Figure 10. *Expression of CD44 and Slug Markers in ICN1-shN3 Cells.*

Western blot analysis using the antibodies CD44 (marker for stemness) and Slug (marker for EMT) shows an absence of expression in the Notch-3 knockdown cell line.

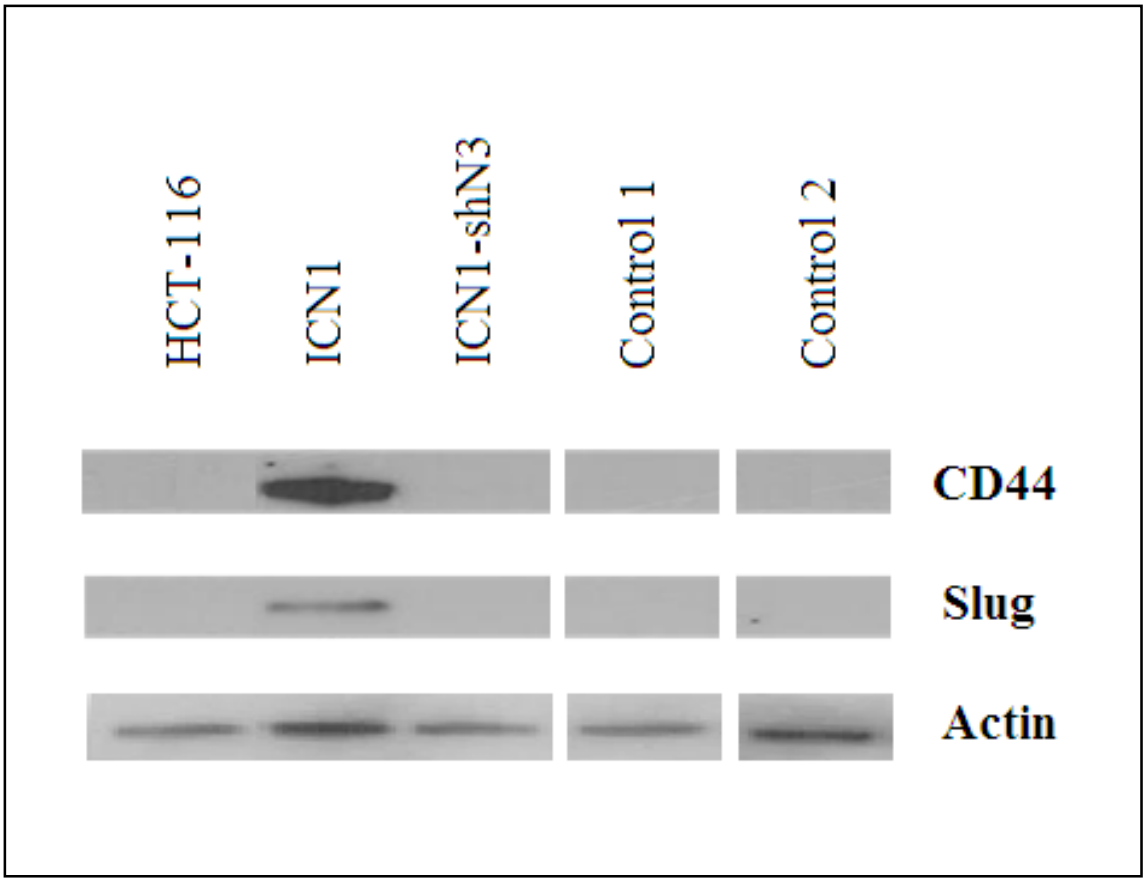


Figure 10. *Expression of CD44 and Slug Markers in ICN1-shN3 Cells.*

Figure 11. *Effect of Notch-3 on Plating Efficiency and Colony Size.*

Single cells from the HCT-116, ICN1, and ICN1-shN3 cell lines were plated into 96-well plates. The number of colonies and the colony size were determined after three weeks of culture using microscopic analysis and the MTT assay. Colonies were grouped into small, medium or large size. *P<0.05; ** P<0.01

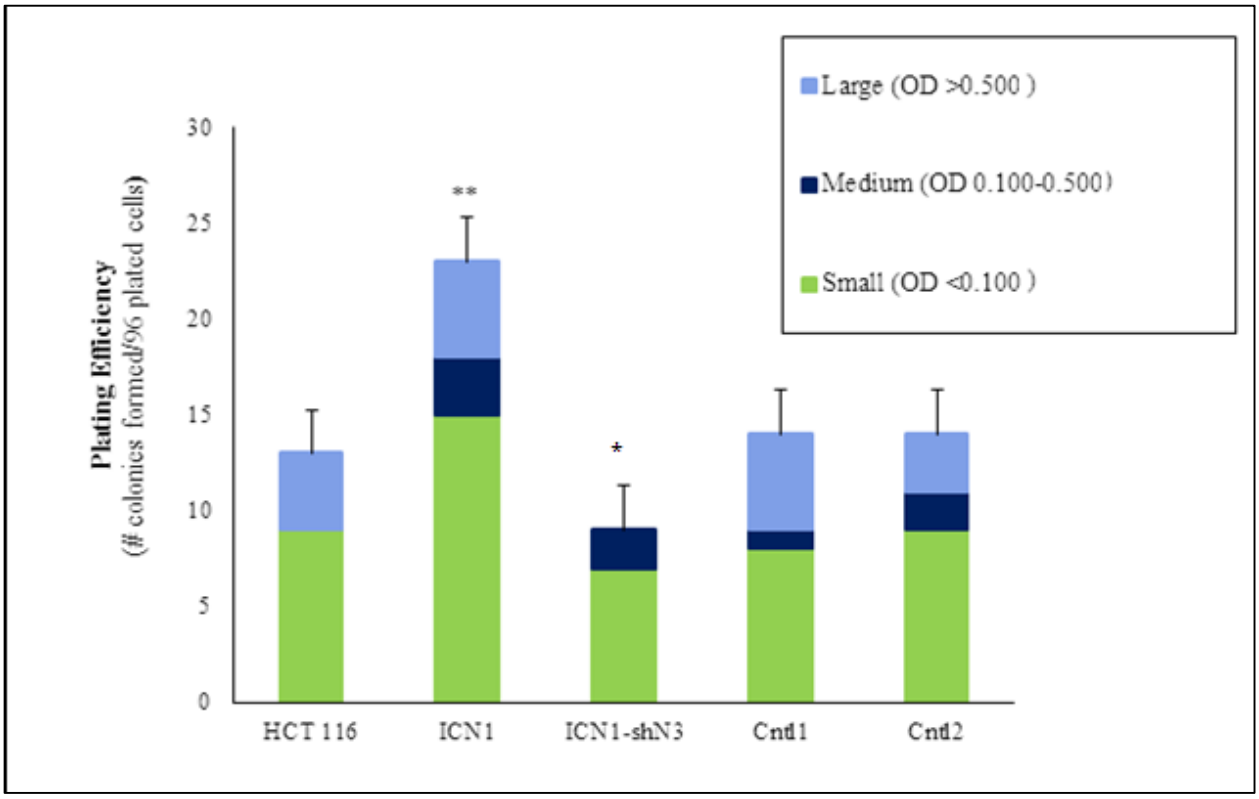


Figure 11. *Effect of Notch-3 on Plating Efficiency and Colony Size.*

We found that the ICN1-shN3 had the greatest number of small size colonies compared to each of the other cell lines *(Fig. 11)*. Unlike the other cell lines studied, the ICN1-shN3 cells did not have any large colonies present.

Duplication Time of ICN1-shN3 Cells

Assessing the dividing time as described in *Methods*, we found that the ICN1-shN3 cell line had a significantly longer duplication than the parental HCT-116 cells or the other control groups (*Table 1)*.

Migration of the ICN1-shN3 Cells

The migratory abilities of the ICN1-shN3 cells were assessed using the wound repair assay (*Fig. 12*). We observed that the ICN1 cells had a significantly greater wound repair ability than the ICN1-shN3 following 24 hours of the initial wound ($P<0.05$).

Colosphere Formation Ability of the ICN1-shN3 Cells.

In an anchorage-independent assay as described in *Methods*, cells from lines HCT-116, ICN1, ICN1-shN3 and the relative controls were plated on soft agar to prevent adhesion to the bottom of the culture dish and to establish an environment conducive to colosphere formation. The number of colonies present for each of the cell lines was determined by low power microscopy after 10 days of growth. We found that by targeting the Notch-3 receptor, there was a significant decrease in colosphere formation by the ICN1-shN3 cells as compared to the ICN1 ($P<0.01$), control, and parental ($P<0.05$) cell lines (*Fig. 13)*. Additionally, colosphere formation was 2.25-fold higher in ICN1 cells compared to the parental cell line. This difference was larger

(3.5-fold) in medium size colospheres. Overall, the ICN1-shN3 cells showed a 35% reduction in colosphere formation compared to the ICN1 cells (*Fig. 13)*.

Table 1. *The Effect of Notch-3 on Duplication Time*

Duplication time of the HCT-116, controls, ICN1, and ICN1-shN3 cells were calculated after 24 hours in culture. *$P<0.05$

Table 1. *Effect of Notch-3 on Duplication Time*

Cell Line	**Dividing Time (Hours)**
HCT-116	20 ± 0.7
ICN1	21 ± 0.3
ICN1-shN3	22 ± 0.3*
Control	20 ± 0.4

Figure 12. *The Role of the Notch-3 Receptor in Migration.*

Migration ability of the ICN1 and ICN1-shN3 cells was determined by a wound healing assay. Wound repair was measured 24 hours post injury.

* $p<0.05$

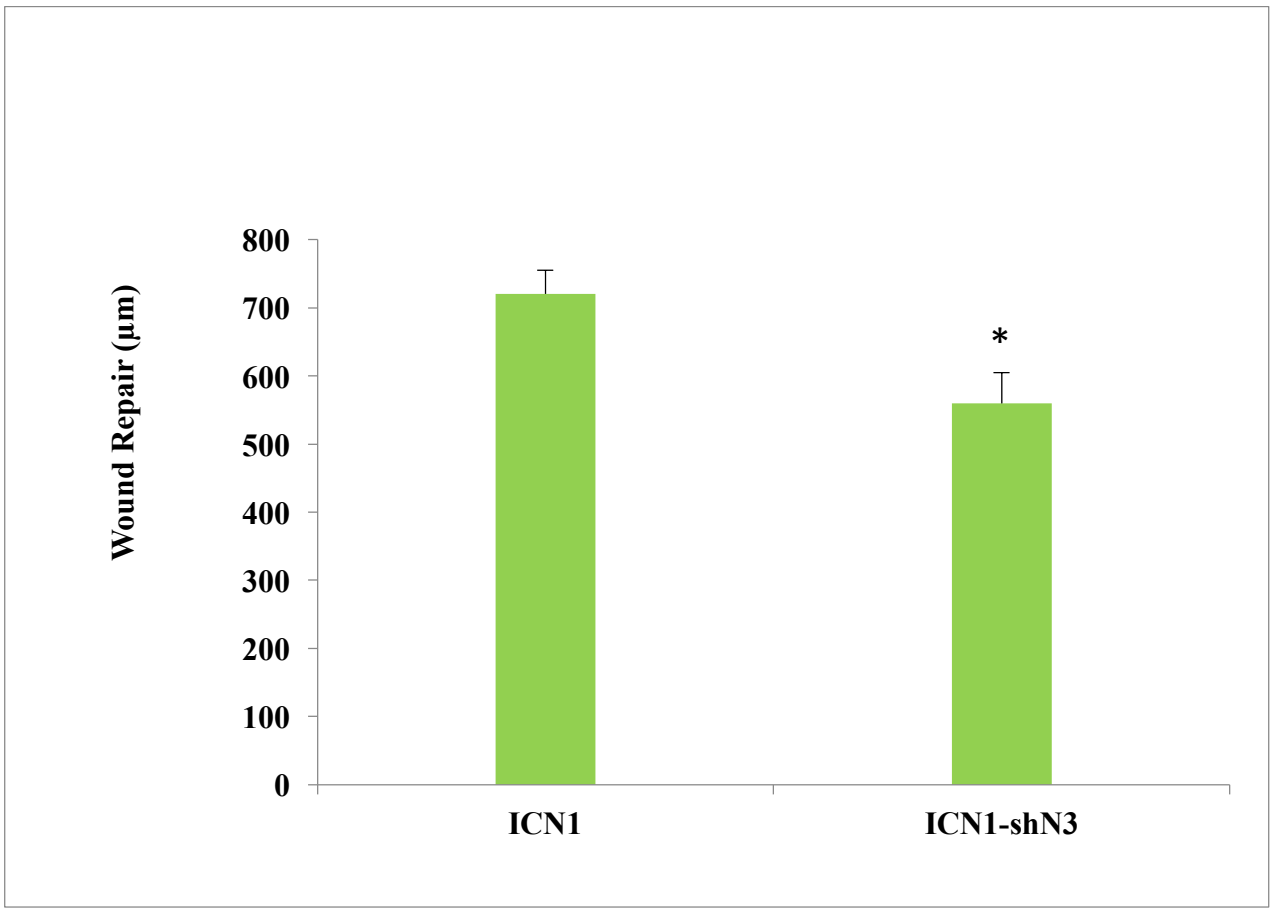


Figure 12. *The Role of the Notch-3 Receptor in Migration.*

Figure 13. *Effect of Notch-3 on Anchorage-Independent Growth.*

Soft agar assays with HCT-116, ICN1, ICN1-shN3 and control cell lines were used. The number of colonies formed in an anchorage independent environment was determined for each of the cell lines via low power microscopy. Cells were grown for 10 days before being counted.

* $p<0.05$; ** $p<0.01$

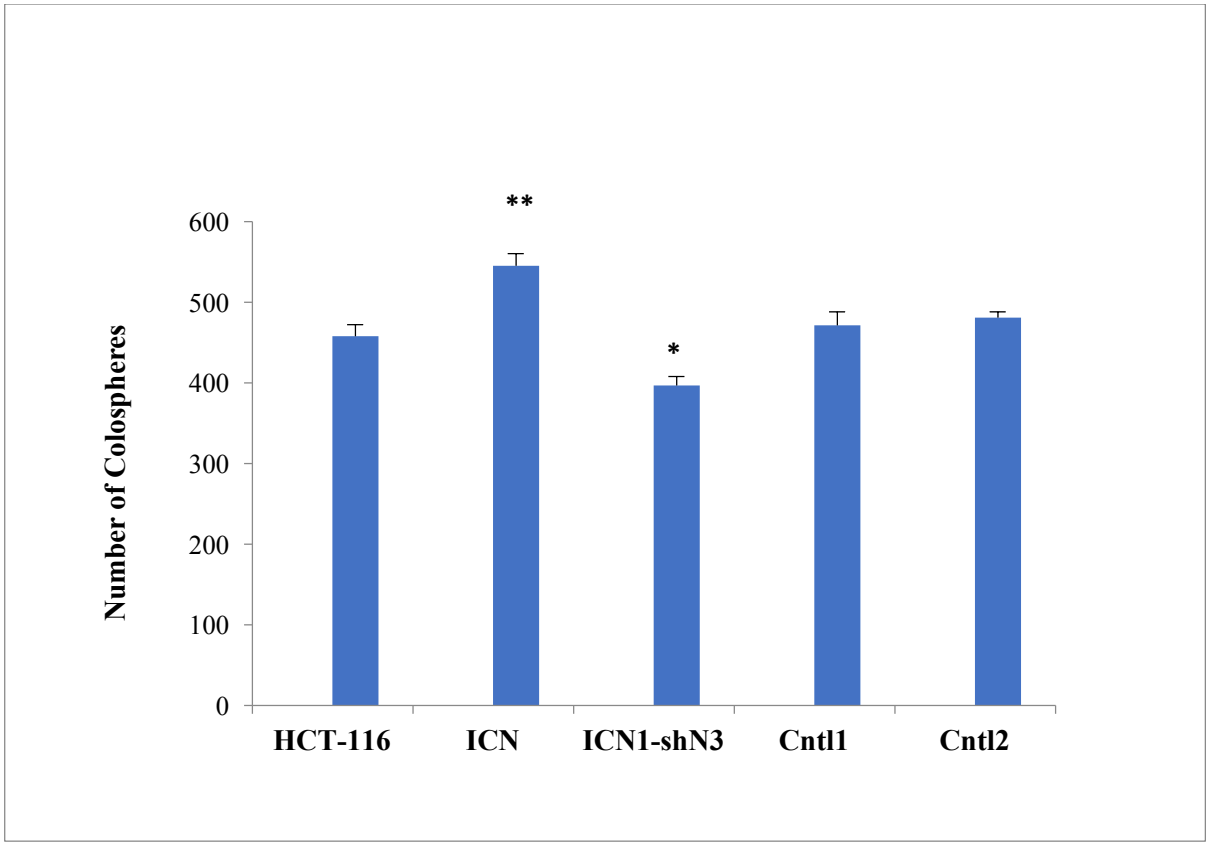


Figure 13. . *Effect of Notch-3 on Anchorage-Independent Growth.*

DISCUSSION

There is overwhelming evidence that Notch signaling plays a significant role in CRC. However, in order to develop new therapies, it is necessary to fully identify the mechanisms of Notch signaling and how they are specifically dysregulated in CRC. In CRC and other cancers, both Notch-1 and Notch-3 have been positively associated with poor survival rates, tumor formation and resistance, and metastasis (Ozawa et al., 2014). Although it has been found that Notch receptor expression (notably Notch-1) is associated with CRC development, little is known about Notch-1's interactions with the other receptors and ligands. Recently, there has been further investigation of the significance of the Notch-3 receptor. In one particular study, Notch-3 is classified as a possible prognostic marker and/or therapeutic target (Ozawa et al., 2014). This group later determined that when Notch-1 and Notch-3 were expressed simultaneously, there was a significant ($P<0.01$) decrease in survival rate compared to those who did not have both receptors expressed (Ozawa et al., 2014). This may even suggest that the Notch-1 and -3 receptors have an additive role in CRC (Ozawa et al., 2014). Results from our group's previous studies have also indicated an association between Notch-1 and Notch-3.

Presently, our group is studying how the Notch-3 receptor mediates Notch-1 induced tumorigenesis in HCT-116 human colorectal carcinoma cells. The ICN1-shN3 cell line, which overexpresses ICN1 and is null for the Notch-3 receptor, was constructed to analyze the Notch-3 receptor specifically (see *Methods)*. The proposed model *(Fig. 5)* laid the framework for the studies conducted in this project.

As the process of EMT occurs, the surface and shape of the cell changes dramatically upon rearrangement of the cytoskeleton. It has previously been recorded that cells with constitutively active Notch-1 exhibit an altered morphology as compared to the parental cell line

(Chappell et al., 2005). In this particular study, cells with upregulated Notch-1 expression exhibited a more rounded morphology, and tended to aggregate in syncytia, whereas the controls and parental cells formed hair-like projections of filopodia along the perimeter allowing for migration (Chappell et al., 2005). Upon assessing the morphological properties of the parental and ICN1 cell lines in our studies, it was evident that these characteristics agree with the published results mentioned above. The spindle-like, rounded shapes of the ICN1 cell line support the claims that upregulated Notch-1 signaling induces stemness. However, because the Notch-3 receptor mediates this mechanism, eliminating it would limit the cell's morphological transformation. The images obtained of the ICN1-shN3 cells concur with the above findings; the morphology of the ICN1-shN3 line appears to reflect a phenotype that shares characteristics of both the HCT-116 and the ICN1 cell lines.

In addition to analysis of morphological traits, the differences in the biochemical properties between the ICN1 and ICN1-shN3 cell lines were investigated. Two notable markers of interest are Slug (a transcriptional repressor of E-Cadherin) and CD44 (a cell surface antigen involved in tumor metastasis). Slug prompts the onset of EMT by directly inhibiting the transcription of E-Cadherin, a cell-cell adhesion molecule (Shao et al., 2015). When functional, E-Cadherin is responsible for maintaining the epithelial state of the cell and can serve as a tumor suppressor (Shao et al., 2015). In addition to oncogenic properties of Slug, a clear association has been established between CD44 expression, the enhancement of EMT, and the invasiveness of CRC (Heerboth et al., 2015). Other studies investigating the role of CD44 have noted that it is highly expressed in primary and metastatic CRC and minimally expressed in normal tissues (Cho et al., 2012). Additionally, CD44 interacts closely with cytoskeletal component, and plays an influential role in tissue remodeling (i.e. migratory response to injury) (Cho et al., 2012). These

previous reviews have facilitated our interpretation of the Western blot shown in *Figure 10.* In these results, it is clear that the omission of Notch-3 eliminates the expression of the Slug and CD44 markers. This indicates that the ICN1-induced signaling pathway relies on the function of Notch-3 to synthesize these EMT and stemness markers.

Additional factors contributing to the progression of stemness, EMT, and tumorigenesis are cellular evasion of apoptosis and the ability to thrive independent of their tissue of origin. When cells become detached or inappropriately positioned within a tissue, anoikis (cell-detachment-induced-apoptosis) is triggered, which arrests the cell cycle and eventually causes caspase-mediated cell death to occur (Guadamillas et al., 2011). One of the hallmarks of cancer cells is developing the ability to overcome anoikis and adapt to an environment other than that of their tissue of origin. The combination of anoikis resistance and anchorage-independence permits tumor cells to expand and invade adjacent tissues, travel through the blood stream, and take root at a secondary location (Vinson et al., 2015). In our studies, an anchorage-independent assay was performed with the parental, control, ICN1, and ICN1-shN3 cell lines. The colosphere-formation ability of each line was determined by the number of colospheres formed in the soft agar after 10 days. Because we found that the ICN1-shN3 cells had reduced colosphere formation ability compared to the controls, parental line, and the ICN1 cells, we were able to conclude that the Notch-3 receptor possessed a distinct role in the ability to form colospheres.

Conclusions regarding cellular properties in CRC were formed by studying the single-cell proliferation of the parental, controls, ICN1, and ICN1-shN3 cell lines. The results from the cell proliferation assay indicated that the ICN1 cells containing the functional Notch-3 receptor formed colonies that were larger in both size and number than the ICN1-shN3 cells. Thus, we

concluded that if the signaling pathway in ICN1 cells is interrupted by blocking the Notch-3 receptor, a decrease in proliferative capacity will occur. Likewise, the conclusions drawn from the dividing time assay results further support the hypothesis. When the parental and control lines were compared to the ICN1-shN3 cells, it was noted that the dividing time was longer for those cells lacking the functional Notch-3 receptor. Longer dividing time is indicative of a less aggressive phenotype.

The assessments of cellular migration and wound healing indicated that the ICN-shN3 cells showed a decreased ability to heal and regenerate cell growth from the wound site when compared to the ICN1 cells. Combined with the results from the plating efficiency and anchorage independent assays, which show that ICN1 cells have an increased tendency over the ICN1-shN3 cells to form colonies and colospheres, these assays collectively support the proposed mechanism described in *Figure* 14 that Notch-1-induced tumorigenesis in human colorectal carcinoma cells is mediated by Notch-3.

For over a decade, the Notch signaling pathway has been a focus in CRC research (Ntziacristos et al., 2014). However, given that the pathway is responsible for normal tissue differentiation and regeneration, generating a therapy targeting only the cancerous cells poses a significant challenge (Ntziacristos et al., 2014). Future directions in developing Notch-targeted therapies for CRC will rely on developing a complete understanding of the Notch mechanism. In particular, further identifying the specificity of Notch receptors and ligands, their downstream targets, as well as the surrounding microenvironment, will enable therapies to use antibodies and other small molecule inhibitors to target only those relevant molecules (Ntziacristos et al., 2014).

Figure 14. *Proposed Model*

Notch-1 signal upregulates the Jagged-1 ligand, which in turn activates the Notch-3 receptor, leading to increased CD44 and Slug expression, ultimately resulting in EMT.

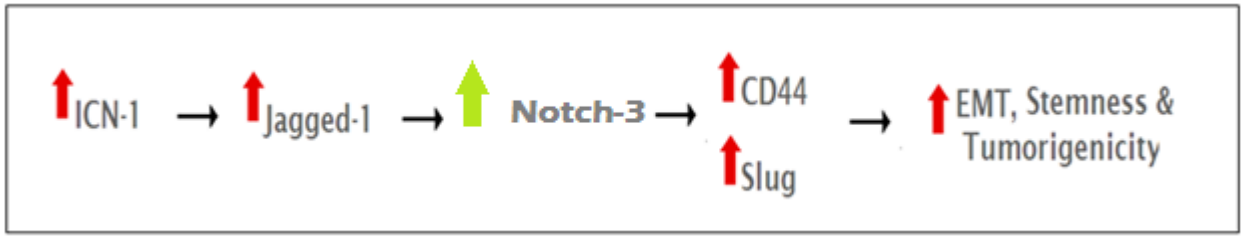


Figure 14. *Proposed Model*

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