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Notch-1 Promotes Stemness and Epithelial to Mesenchymal Transition in Colorectal Cancer

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ABSTRACT

Colorectal cancer (CRC) is the third leading cause of cancer death in the United States, resulting in an average of 50,000 deaths per year. Surgery and combination chemotherapy comprise current treatment strategies. However, curative options are limited if surgery and chemotherapy are unsuccessful. Several studies have indicated that CRC aggressiveness and potential for metastatic spread are associated with the acquisition of stem cell like properties. The Notch-1 receptor and its cognate signaling pathway is well known for controlling cell fate decisions and stem-cell phenotypes. Alterations in Notch receptors and Notch signaling has been reported for some colon cancers. Herein, we examine a potential role for Notch-1 signaling in CRC. In CRC patient samples, Notch-1 expression was increased in colon tumor tissue as compared with normal colon tissue. Retroviral transduction of constitutively active Notch-1 (ICN1) into the colon tumor cell line HCT-116 resulted in increased expression of the EMT/stemness associated proteins CD44, Slug, Smad-3, and induction of Jagged-1 expression. These changes in ICN1 expressing cells were accompanied by increased migration and increased anchorage independent growth by 2.5-fold and 23%, respectively. Experiments with the pan-Notch inhibitor DAPT, and soluble Jagged-1-Fc protein provided evidence that Notch-1 signaling activates CD44, Slug, and Smad-3 via a cascade of other Notch-receptors through induction of Jagged-1 expression. These data indicate a key role for Notch signaling in the phenotype of CRC and suggest that targeting of Notch signaling may be of therapeutic value in colon cancers. J. Cell. Biochem. 116: 2517–2527, 2015.

KEY WORDS: COLON CANCER; EMT; NOTCH SIGNALING; STEMNESS

Abbreviations: CMML, chronic myelomonocytic leukemia; CRC, colorectal cancer; DAPT, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; LMP, low melting point; MAML-1, mastermind like protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NECD, notch extracellular domain; PBS, phosphate buffer saline; T-ALL, T-cell acute lymphoblastic leukemia.

Authors' Contributions

Alexander W. Fender performed the vast majority of the experiments under the supervision of Dr. Sigounas. He also participated in data analysis, experimental design and writing of the manuscript.

Jennifer M. Nutter performed the transwell migration assays, as well as certain steps of the retroviral transduction and selection of cells under the supervision of Dr. Bertrand. She also participated in data analysis and experimental design.

Timothy L. Fitzgerald supervised the process of tissue procurement and consenting patients. In addition, he participated in discussions of data analysis and experimental design.

Fred E. Bertrand performed the retroviral insertion of the Notch-1 and vector control in transfected HCT-116 cells, as well as some of the Western blot analysis presented. He also participated in data analysis and writing of the manuscript.

George Sigounas initiated the project and participated in all aspects of the study (idea conception; experimental design; data analysis; writing of the manuscript; etc.).

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Colorectal cancer (CRC) is one of the leading causes of cancer mortality and kills an average of 50,000 people each year in the United States [American Cancer Society, 2014; Siegel et al., 2014]. Current treatments include tumor removal via surgery and combination chemotherapy which have reduced the number of deaths from colorectal cancer. However, there are limited available treatment options if surgery and chemotherapy are unsuccessful. Possibilities for new therapeutics include inhibition of oncogenic signaling pathways and forced activation of tumor suppressor pathways. Before implementing either of these new possibilities, the function of signaling pathways in colorectal cancer must be further characterized [Bertrand et al., 2012].

The Notch signaling pathway is classically known for regulating cell fate decisions between cells expressing the receptor and other cells expressing the ligand. However, alterations in Notch are associated with tumorigenesis [Klüppel and Wrana, 2005; Roy et al., 2007]. Activating mutations in Notch-1 are associated with T-ALL (T-cell acute lymphoblastic leukemia), breast cancer and have been reported in colon cancer [Klüppel and Wrana, 2005; Roy et al., 2007]. In contrast, inactivating mutations of Notch are associated with CMML (chronic myelomonocytic leukemia), skin cancer, and head and neck squamous cell carcinoma. Thus, Notch can function as both an oncogene and a tumor suppressor depending upon cellular context [Bertrand et al., 2012].

The Notch pathway in humans is composed of four receptors, Notch-1, -2, -3, and -4 along with five ligands, Jagged-1, Jagged-2, Delta (DLL)-1, DLL-3, and DLL-4 [Klüppel and Wrana, 2005; Roy et al., 2007; Bertrand et al., 2012]. The Notch receptors are composed of an extracellular (NECD, Notch extracellular domain), a transmembrane, and an intracellular domain (NICD, Notch intracytoplasmic domain) [Hori et al., 2013]. Upon binding of the Notch receptor to one of the ligands, a cascade of proteolytic cleavages takes place mediated by a metalloprotease followed by γ-secretase activity. This results in the release of an intracytoplasmic Notch (ICN) fragment which translocates to the nucleus. There, it associates with CBF-1 (also known as RBP-Jk, recombination signal sequence binding protein Jk) and MAML-1 (Mastermind Like Protein 1) which are part of a transcription complex [Hori et al., 2013]. Of the ligands, DLL-4 is associated with angiogenesis, whereas Jagged-1 seems to be associated with metastasis in a variety of cancers including prostate, breast, and colon. Jagged-1 induced Notch signaling is required for breast cancer metastasis [Sethi et al., 2011]. In colon, Jagged-1 appears to be a TGF-βresponsive gene [Sonoshita et al., 2011; Calon et al., 2012]. Thus, the Notch-receptor signaling pathway may be part of a regulatory loop that helps to shape the TGF-β driven tumor microenvironment.

The role of Notch signaling in the structure and function of the normal gastrointestinal system has been well-documented [Kemper et al., 2010; Fre et al., 2011; Miyamoto and Rosenberg, 2011; Vooijs et al., 2011; Geissler and Zach, 2012; Vaiopoulos et al., 2012]. However, the contribution of Notch signaling in colorectal cancer has not been thoroughly studied, although there is some data suggesting an oncogenic role. In one study, a gene array analysis revealed that Notch-1 and its target Hes-1 were expressed more in advanced tumors than low grade tumors [Meng et al., 2009]. The array also showed a decrease in levels of Numb, a Notch antagonist, in high grade tumors. Another study revealed active Notch signaling in colon tumors via in situ hybridization, though a correlation between patient mortality and Hes-1 expression was not detected [Reedijk et al., 2008]. There are current ongoing studies assessing the role of Notch signaling specifically in cancer stem (or initiating) cells [Bertrand et al., 2012].

The ability to undergo epithelial to mesenchymal transition (EMT) and migrate to embryological sites during organogenesis is a fundamental biological property of stem cells. For example, EMT is essential for the formation of the neural crest in embryogenesis. EMT is also closely associated with increased stemness in tumors. It has been proposed that for cancer stem cells to dissociate from a primary tumor and to circulate in the blood they must undergo EMT [Yang and Weinberg, 2008]. Many models also identify EMT as the first process in metastasis [Iwatsuki et al., 2010; Raimondi et al., 2011; Scheel and Weinberg, 2012]. Hallmarks of EMT include increased expression of the transcription factors Snail, Slug, and Twist, which play key regulatory roles in the EMT and hence the metastatic process. Increased expression of mesenchymal intermediate filament, vimentin and loss or reduced expression of the tight junction protein E-cadherin is also observed during EMT [Gao et al., 2012].

Herein, we observed that Notch-1 expression was highly expressed in the tumors of patients with colorectal cancer as compared with their normal counterparts. These data indicate that the Notch pathway and likely Notch signaling is elevated in CRC cells, and suggests a mechanistic role for Notch in colon tumorigenesis. Based on these data and the literature [Meng et al., 2009; Reedijk et al., 2008], we postulated that Notch-1 activation drives stemness properties and increases EMT in colorectal cancer. To test this hypothesis, the colon tumor cell line HCT-116 was stably transduced with a retrovirus expressing a constitutively active intracytoplasmic fragment of human Notch-1 (ICN1). This resulted in alterations in anchorage dependent growth, proliferation, migration and wound healing, and was accompanied by induction of the Notch-ligand Jagged-1, CD44, Slug, and Smad-3.

MATERIALS AND METHODS

PATIENT SAMPLES AND SPECIMEN PROCESSING

Fresh or frozen tissue from patients with colorectal cancer was available via the North Carolina Tissue Consortium at East Carolina University. At the time of the operative procedure, the tissue was procured via a University and Medical Center Institutional Review Board (UMCIRB) approved protocol. Under sterile conditions, both normal and cancerous tissue was obtained by the pathologists. The epithelial areas of the surgically removed normal and neoplastic colon tissue were separated from the adipose tissue, connective tissue, and blood vessels. The tissue was then minced and processed for Western blot analysis using standard protocols [Sigounas et al., 2010]. For the isolation of primary cells, the minced tissue was digested in medium containing insulin (10 μg/mL), hyaluronidase (100 U/mL), and collagenase (200 U/mL). The digested tissue was centrifuged at 300*g* for 5 min and the supernatant was removed. The pellet was washed 2× with PBS (phosphate buffer saline). The single cells derived from the digested normal or neoplastic colon tissue

were then used for cell column purification, protein analysis, gene transfer, and/or cultures [Sigounas et al., 2010].

CELL LINES AND CULTURE

The experiments described in this study were conducted with the colon cancer cell line HCT-116 (ATCC, Manassas, VA). In order to perform our studies, we generated HCT-116 cells that express constitutively active Notch-1 (ICN1 cells) and HCT-116 cells which expressed the green fluorescent protein (GFP) protein alone (Vector cells). HCT-116 cells were transduced with an IRES-GFP retrovirus expressing human ICN1 [Chappell et al., 2005], as previously described [Pui et al., 1999]. Briefly, The ICN1 retrovirus was packaged by co-transfection with a plasmid expressing the amphotropic viral coat in 293 cells. Supernatant containing packaged viral particle was collected and overlaid onto HCT-116 cells. GFP-positive cells expressing constitutively active Notch-1 were then collected by FACS sorting.

The parental HCT-116, the Vector alone and the ICN1 cell lines were grown in T25 flasks containing McCoys' 5A medium (Life Technologies, Carlsbad, CA) supplemented with glucose, 10% fetal bovine serum (FBS) (Atlanta Biologicals) and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin) (Life Technologies). Before reaching 90% confluence, the cells were trypsinized and passaged.

CELL DIVIDING TIME AND COLONY GROWTH ASSAYS

Twenty thousand cells from the HCT-116, Vector alone and ICN1 cell lines were plated into individual T-25 flasks containing complete medium. After six days, the cells were fully trypsinized and counted with a hemacytometer (Cambridge Instruments Inc) to determine the total number of cells in the flask. The duplication time was calculated using the following formula: $d = t^*Ln(2)/Ln(N_t/N_0)$, where N_t is the number of cells at the time of counting, N_0 is the initial amount of cells, t is the hours of growth, and d is the duplication time in hours.

To assess colony growth, single cells from HCT-116, Vector alone and ICN1 cell lines were plated into 96 well plates using the FACSVantage SE cell sorter (Becton Dickinson). The number of cells in each single cell-derived colony was determined after three days of culture.

WOUND HEALING ASSAY

Cells were grown in six well plates until 90–95% confluent. Three vertical scratches from the top to bottom of each well were made with a 200 μL pipette tip (Corning, NY) and the width of each scratch was measured in three places using a microscope with scale in the ocular. After 24 h, the scratches were measured again. Wound repair was determined by subtracting the second measurement from the first.

TRANSWELL MIGRATION ASSAY

Polycarbonate 8.0 μm membrane transwells (Costar, Washington DC) were placed in 24 well plates. The lower chamber was filled with RPMI (Life Technologies) plus 20% fetal calf serum (Sigma, Saint Louis, MO). Cells were plated in the top chamber in RPMI without serum. After 24 h of incubation at 37°C, the cells that migrated through the transwell membrane were fixed in 70% EtOH (Pharmco-AAPER, Brookfield, CT), stained with 0.2% crystal violet (Sigma), and counted using a 100× field [Sui et al., 2014].

ANCHORAGE INDEPENDENT GROWTH ASSAY

Soft agar assays were performed to determine if manipulating the Notch pathway will influence the ability of CRC cells to form colonies under conditions of anchorage independent growth. Increased colony formation in this assay is associated with a more aggressive phenotype and increased metastatic potential. These experiments were performed with the panel of HCT-116, Vector, and ICN1 cells. Briefly, six-well plates were coated with a bottom layer of 0.8% LMP (low melting point) agarose (Life Technologies) to prevent cell attachment. Then 5,000 cells were mixed with growth media and LMP agarose to a final concentration of 0.4%. Cells were cultured until colonies were readily visible to the naked eye—typically about ten days. Viable colonies were stained with MTT (Sigma) and counted via low power microscopy.

WESTERN BLOT ANALYSIS

Western blot analysis was performed as previously described [Sigounas et al., 2010]. Briefly, cell pellets were placed in Tris-HCl (20 mM) containing EDTA (2 mM), EGTA (2 mM), 2-mercaptoethanol (10 mM), leupeptin (50 μg/mL), aprotinin (20 μg/mL), pepstatin (10 μM), and phenylmethylsulfonyl fluoride (1 mM) (Sigma). The solution was placed on ice for 15 min and was then centrifuged in 4°C at 14,000 RPM for 15 min. The supernatants were stored at −80° C until use. The proteins were fractionated on anSDS-polyacrylamide gradient gel and then transferred to a PVDF membrane (Bio-Rad). Blots were placed into the appropriate dilution of the primary antibodies such as β-actin, ALDHA1, Hes-1, Jagged1, Smad2/3, E-Cadherin, Notch-1, and CD44 (Cell Signaling, Beverly, MA) as well as Lgr5 (Abgent, San Diego, CA), EpCAM and Slug (Santa Cruz Biotech, Santa Cruz, CA) . After incubation with the primary antibodies, blots were placed into goat anti mouse or goat anti rabbit secondary antibody (Santa Cruz Biotech) for 2 h. Proteins were visualized with chemiluminescence with SuperSignal West Dura Extended Duration Substrate (Pierce) and FluorChem 8,900 visualization system from Alpha Innotech. Quantification of the expressed proteins was performed using the Quantiscan program (Biosoft, Cambridge, United Kingdom).

PURIFIED NOTCH LIGAND

To further verify findings from experiments using retrovirally introduced ICN1, colon cancer cells were cultured in the presence of purified Notch ligands. Purified Jagged-1 ligand was purchased from R&D Systems. Plates were coated with Jagged-1 ligand and then overlaid with colon cancer tumor cells. These cells were then assessed for morphological alterations as well as expression of proteins associated with stemness and EMT.

PHARMACOLOGIC INHIBITION OF NOTCH

Pharmacologic inhibition of Notch-1 signaling has been widely studied as a potential therapeutic approach to certain cancers and also Alzheimer's disease. To confirm our findings with overexpressed Notch-1 and to determine the mechanistic nature of these observations, cells were treated with the Notch inhibitor DAPT (10 μM) for various time intervals. Loss or inhibition of Notch-1 signaling was measured by decrease or loss of Hes-1 expression, a well-defined Notch target gene.

STATISTICAL ANALYSIS

In order to evaluate differences between groups, variables were analyzed with Student′s *t*-test [Fitzgerald et al., 2014]. The Student′s *t*-test was two-sided and was 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 RECEPTORS ARE UNIQUELY EXPRESSED IN TUMORS FROM COLORECTAL CANCER PATIENTS

We examined Notch receptor protein expression in colon cancer tissues and colon normal tissues from six patients undergoing a colectomy. Notch-1 was highly expressed in the CRC tumors of these patients as compared with their normal counterparts. Figure 1A presents Notch-1 expression in normal (N) versus tumor (T) cells from four of these patients (Fig. 1A). In other studies, we examined Notch receptor and ligand protein expression in primary colon cancer cells and normal colon epithelial cells derived from a colorectal cancer patient. Notch-1 expression was highly expressed in the CRC tumor cells from this patient as compared with their normal primary epithelial counterparts (Fig. 1B). Although preliminary due to the limited sample size, these data suggest that the Notch pathway and likely Notch signaling is elevated in CRC cells, consistent with earlier reports by Meng et al. (2010). We thus sought to establish a cell line model in which the biological basis forNotch-mediated effects on colon tumor cells could be studied.

GENERATION OF HCT-116 CELLS EXPRESSING CONSTITUTIVELY ACTIVE NOTCH-1

HCT-116 cells were transduced with retrovirus expressing constitutively active Notch-1 (ICN cells) or an empty vector as a control (Fig. 2A). Transduced cells were collected by FACS sorting on the basis of GFP expression (Fig. 2B), and subsequently analyzed by fluorescence microscopy (Fig. 2C–F). Confirmation of the overexpression of the intracytoplasmic domain of Notch-1 was determined by Western blot analysis (Fig. 2G).

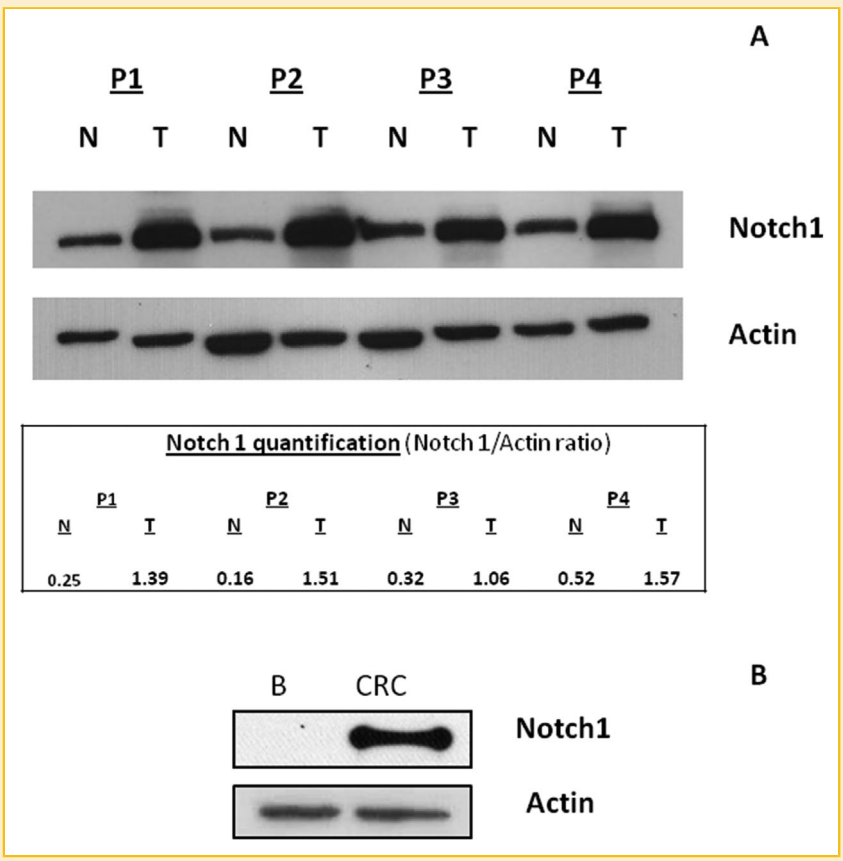

Fig. 1. (Panel A) Notch-1 proteins are highly expressed in colon tumors. Colon tumor tissue and colon normal epithelial tissue were isolated from four patients undergoing colectomy. Western blot analysis was performed on whole cell protein lysates separated by SDS-PAGE. Blots were probed with the indicated antibodies. Numeric data insert shows Notch 1 protein level quantification in the respective tissues. N, normal tissue; T, colorectal tumor tissue; P1, patient 1; P2, patient 2; P3, patient 3; P4, patient 4. (Panel B) Notch-1 proteins are expressed only in colon tumor-derived epithelial cells. Colon tumor epithelial cells and colon normal epithelial cells were isolated from a patient undergoing colectomy. Western blot analysis was performed on whole cell protein lysates. Blots were probed with the indicated antibodies. B, normal cells; CRC, colorectal tumor cells. Levels of actin were used as internal controls.

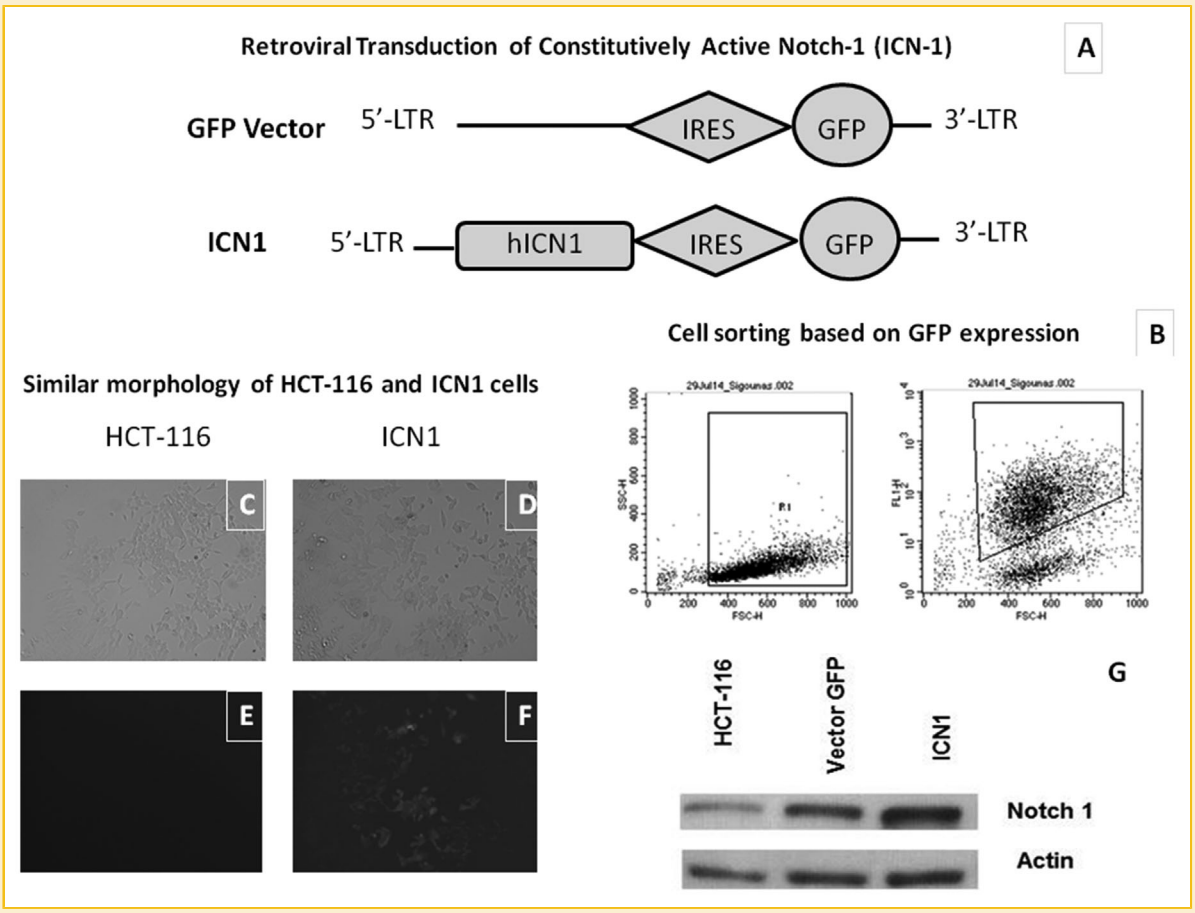

Fig. 2. Overexpression of Notch-1 in colorectal cancer cells. (Panel A) Retroviral constructs. A construct containing the intracytoplasmic domain of Notch 1 (ICN-1) and the green fluorescent protein (GFP) gene was inserted into the DNA of HCT-116 cells using a retroviral vector. A construct containing the GFP gene alone was also inserted into HCT-116 cells to create the Vector alone cell line used as another control. (Panel B) Cell sorting of retrovirally- transduced cells. Cells expressing GFP were separated from cells lacking GFP expression to create the ICN1 and Vector alone cell lines using the Becton Dickinson FACSVantage SE cell sorter. ICN1-transduced cells expressing GFP (gated region of right picture of Fig. 2B) were separated from cells negative for GFP expression (ungated region of right picture of Figure 2B) to form the working ICN1 line. (Panels C–F) Microscopic analysis of colorectal cancer cells expressing a constitutively active Notch-1 and GFP. Pictures of HCT-116 (Panels C and E) and ICN1 (Panels D and F) cells were taken with the EVOS microscope in white (Panels C and D) and green fluorescent (Panels E and F) light at 10× magnification. (Panel G) Overexpression of the intracytoplasmic domain of Notch-1. Proteins from whole cell lysate from parental HCT-116 cells, vector control cells and ICN1 cells assessed by Western blot analysis with the indicated antibodies.

ROLE OF NOTCH SIGNALING ON CELL DIVIDING TIME, COLONY SIZE, AND WOUND REPAIR

Six days following plating of cells into T-25 flasks, the total number of cells was determined and the duplication time was calculated as described in Methods. We found that cells overexpressing the intracytoplasmic domain of Notch-1 divided more slowly and their duplication time was significantly longer by almost 3 h than the parental cell line, 20.3 ± 0.07 and 17.8 ± 0.15 h, respectively (P-value $= 0.001$). The utilized vector and expression of GFP did not affect cell division and cell counting revealed no significant difference in duplication time of GFP alone expressing cells (17.9 ± 0.19 h) when compared to the parental HCT-116 group ($P > 0.05$).

In other studies assessing the size of single cell-derived colonies, we found that ICN1 cells produced smaller colonies compared to the parental HCT-116 or the vector alone transfected cells ($P < 0.001$) (Fig. 3A). These results are in concordance with a slower dividing time of ICN1 cells.

Assessing the ability of cells expressing increased levels of ICN1 to repair wounds, we observed that the parental cells were more proficient by 1.5-fold in repairing wounds as compared with cells overexpressing the intracytoplasmic domain of Notch-1 24 h following injury ($P < 0.0001$) (Fig. 3B). Expression of GFP did not significantly affect the wound repair capacity of the vector alone transduced colon cells ($P > 0.05$) (Fig. 3B).

NOTCH-1 OVEREXPRESSION AFFECTS MIGRATORY PROPERTIES OF COLORECTAL CANCER CELLS

To assess the effect of constitutively active Notch-1 on cell migration, cells were placed in transwells on top of media containing chemoattractant. After 24 h of incubation, the cells that migrated through the transwell were fixed, stained, and counted. We found that overexpression of Notch-1 induced a 2.5-fold increase in cell migration compared to the parental colon cell line (P-value $= 0.003$) (Fig. 3C). GFP expression (vector alone cells) revealed no significant

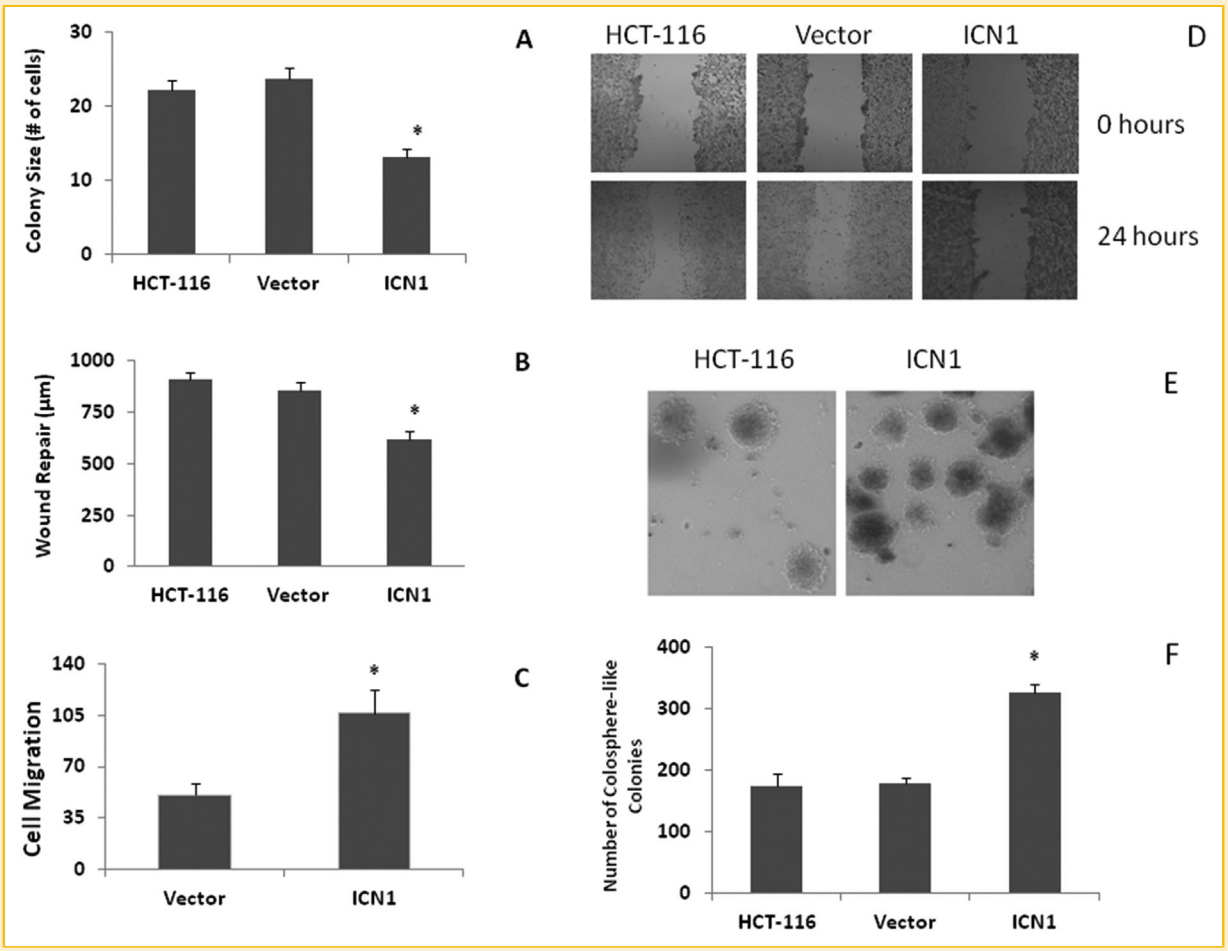

Fig. 3. (Panels A–C) A constitutively active Notch-1 affects biological properties of colon cancer cells. (Panel A) Single cells from HCT-116, vector alone and ICN1 cell lines were plated into 96-well plates using the Becton Dickinson FACSVantage SE cell sorter. The number of cells in each single cell-derived colony was determined after three days of growth. Bars represent means ± SE of cells per colony size. **P*-value < 0.001. N = 6. (Panels B and D) Cells from HCT-116, vector alone and ICN1 cell lines were grown in six-well plates until 90–95% confluent. Three vertical scratches were made and the width of each scratch was measured in three places using a microscope with a ruler in one of the eye pieces. After 24 h, the scratches were measured again in the same places as before. Wound repair was determined by subtracting the second measurement from the first. The bars represent means ± SE. **P*-value < 0.0001. N = 6. (Panel C) Notch overexpression enhances migratory properties of colorectal cancer cells. Cell permeable membranes were placed in 24-well plates on top of media containing the chemoattractant. HCT-116 and ICN1 cells were plated on top of the membranes and after 24 h of incubation, the cells that migrated to the bottom of the well were fixed, stained, and counted. The bars represent mean ± SE of cells trapped on the membrane. **P*-value=0.003. N = 3. (Panels E and F) Notch overexpression increases anchorage-independent growth of colorectal cancer cells. Plates were coated with 0.8% LMP agarose to prevent cell attachment. Five thousand cells from HCT-116, Vector alone and ICN1 cell lines were plated into individual wells and grown in 0.4% agarose mixed with complete medium. After ten days, the cells were stained with MTT and colonies were counted using a microscope (Panel E). The graph (Panel F) represents the average number of colonies ± SE. **P*-value = 0.004. N = 3.

difference in cell migration compared to the parental group (data not shown).

NOTCH-1 OVEREXPRESSION AFFECTS THE ABILITY TO FORM ANCHORAGE-INDEPENDENT COLONIES

We explored the ability of colon cancer cells to grow under anchorage independent conditions using the soft agar assay (Fig. 3E). Microscopic analysis of colonies indicated that ICN1 cells produced 47% more colosphere-like colonies (defined as detached, compact and rounded in appearance) as compared to the parental HCT-116 cell line ten days following plating ($P < 0.004$) (Fig. 3F). However, expression of GFP (Vector alone cells) had no significant effect on the levels of colonies formed ($P = 0.82$) (Fig. 3F).

CONSTITUTIVELY ACTIVE NOTCH-1 ALTERS THE EXPRESSION OF STEMNESS AND EMT RELATED PROTEINS

The expression of proteins associated with stemness, epithelial to mesenchymal transition, and the Notch pathways in response to constitutively active Notch-1 were determined by Western blot analysis. We found that there was differential expression of CD44, Slug, Smad-3, Jagged-1, Hes-1, and E-cadherin in cells over-expressing the intracytoplasmic domain of Notch-1 when compared with the control groups (Fig. 4). CD44 exhibited a several-fold increase in cells expressing the constitutively active Notch-1 as compared to the parental group ($P < 0.01$) (Fig. 4A and B). In addition, a very significant increase of Slug was detected in ICN1 overexpressing cells when compared to the undetectable levels of the control groups (Fig. 4A and B). The levels of Smad-3, Jagged-1, and

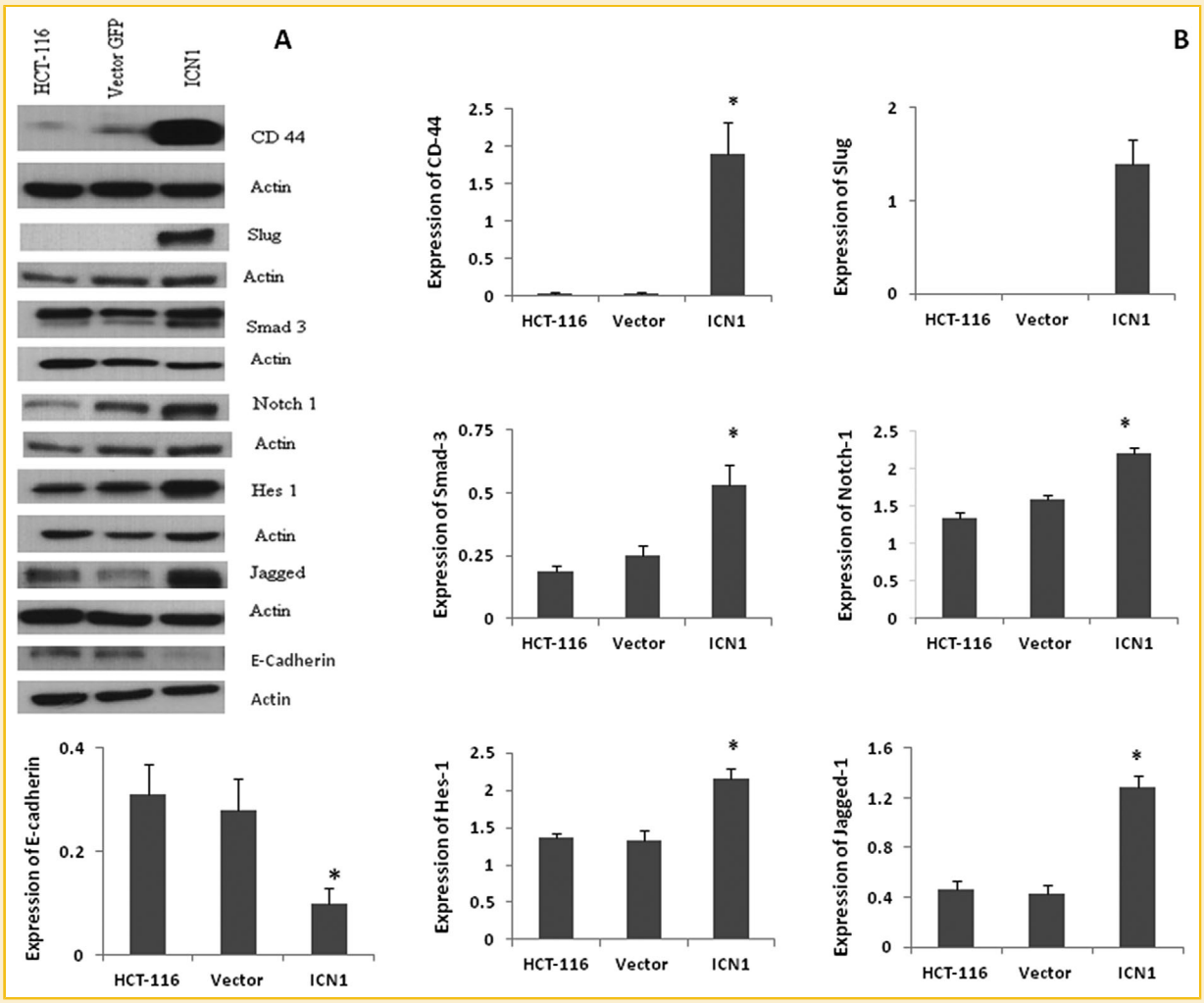

Fig. 4. Protein expression of HCT-116 cells in the presence of constitutively active Notch-1. Proteins from whole cell lysate from parental HCT-116, Vector alone and ICN1 cells were assessed by Western blot analysis with CD44, slug, Smad-3 (lower band), Notch-1, Hes-1, Jagged-1, and E-cadherin antibodies (Panel A). Quantification of the expressed proteins was performed using the Quantiscan program (Panel B). Bars represent means ± SE of protein expression relative to β-actin. *P-value < 0.01–0.002. N = 5.

Hes-1 were 2.8, 2.8, and 1.6 times higher in the ICN1 cells compared to the control group, respectively ($P < 0.01$; $P < 0.002$; $P < 0.002$) (Fig. 4A and B). Meanwhile, E-cadherin was reduced by 3.1-fold in the ICN1 cells than their counterpart controls ($P < 0.01$) (Fig. 4A and B). There was no difference in the expression of the aforementioned protein between the GFP alone expressing cells and the parental group ($P > 0.05$) (Fig. 4A and B). In addition, Western blot analysis indicated that there was an increased expression of ALDH1 in ICN1 cells compared to HCT-116, but this was not statistically significant (data not shown). However, using flow cytometric analysis, we found that ICN1 cells had 9.1% more ALDH1 positive cells as compared to the parental HCT-116 cells and the Vector control.

γ-SECRETASE INHIBITOR DAPT SUPPRESSES THE ANCHORAGE-INDEPENDENT GROWTH OF COLORECTAL CANCER CELLS

Our previous experiments showed that a constitutively active Notch-1 altered the phenotype of colorectal cancer cells, enhanced their anchorage-independent growth, and induced an increase in proteins associated with EMT. To determine whether other members of the Notch family were involved in EMT induction, biological and biochemical properties of the colon cancer cells were investigated following treatment with DAPT, an effective inhibitor of γ-secretase. DAPT treatment induced a decrease in anchorage-independent growth of both HCT-116 ($P < 0.02$) and ICN1 ($P < 0.002$) cells by 31% and 65%, respectively (Fig. 5A and C). Meanwhile, the levels of the colosphere-like structures formed by the parental cell line and the GFP-alone expressing cells were similar (Fig. 5A and B). It is noteworthy to mention that DAPT treatment does not affect the intracytoplasmic domain of Notch-1 in cells expressing a constitutively active Notch-1 since expression of this domain from the retroviral construct is not associated with the functionality of γ-secretase.

PROTEIN EXPRESSION OF DAPT TREATED HCT-116 CELLS IN THE PRESENCE OF CONSTITUTIVELY ACTIVE NOTCH-1

Since constitutively active Notch-1 (ICN1) induced overexpression of transcription factors and proteins associated with developmental

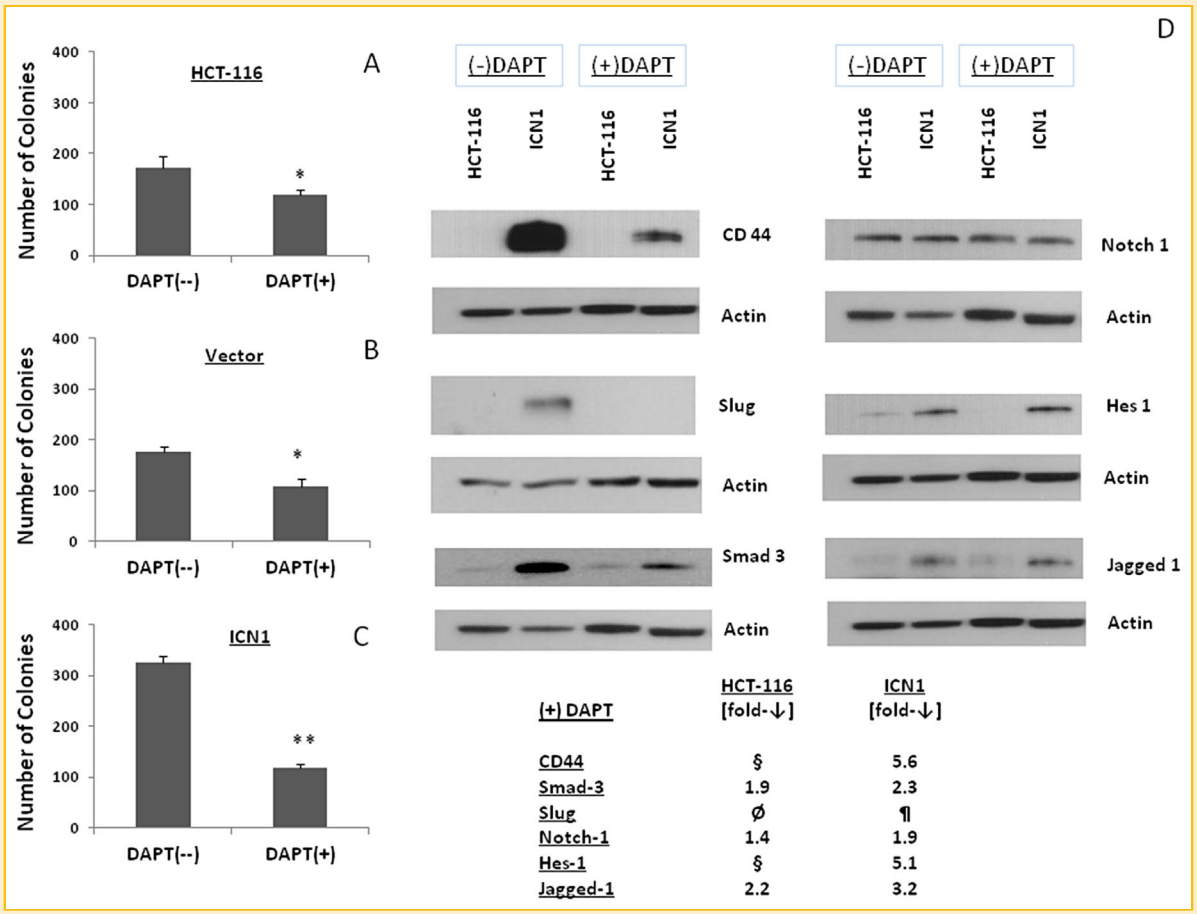

Fig. 5. (Panels A–C) The γ-secretase-inhibitor DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) inhibits anchorage-independent growth of colorectal cancer cells. Five thousand cells from HCT-116, Vector alone and ICN1 cell lines were plated into agarose-coated wells and grown in 0.4% agarose mixed with complete medium in the presence or absence of DAPT. After ten days, the cells were stained with MTT and colonies were counted using a microscope. The graph represents the average number of colonies ± SE. **P*-value < 0.02. N = 3. (Panel D) DAPT modifies protein expression of HCT-116 cells in the presence of constitutively active Notch-1. Proteins from parental HCT-116 cells and ICN1 cells treated with DAPT were assessed by Western blot analysis using the indicated antibodies. Numeric data insert shows protein level decrease following treatment of cells with DAPT. (−) DAPT, without DAPT; (+) DAPT, with DAPT; §, no bands are shown in HCT-116 cells in short film exposures though bands were present in longer film exposures; Ø & ¶, slug was only expressed in DAPT untreated ICN cells.

pathways, we studied whether the intact Notch pathways had a direct effect on the expression of CD44 and Slug. The parental HCT-116 cell line and cells expressing the intracytoplasmic domain of Notch-1 were cultured in the presence or absence of the Notch inhibitor DAPT and the expression of CD44, Smad-3, Slug, Jagged-1, Notch-1, and Hes-1 was determined by Western blot analysis. We found that after 24 h of exposure to DAPT, the parental cells exhibited a several-fold decrease regarding the levels of Hes-1 indicating an effective inhibition of the Notch pathways (Fig. 5D; numerical data insert). On the other hand, Hes-1 decreased by only 5.1-fold in DAPT treated cells expressing ICN1 (Fig. 5D; numerical data inset). Although the relative expression of the assessed proteins was low in HCT-116, DAPT treatment of the parental cells induced a moderate decrease (ranged from 0-fold to 2.2-fold) in proteins such as CD44, Smad-3, and Jagged-1 compared to the untreated control group (Fig. 5D; numerical data insrt), likely indicating a low level of endogenous Notch-1 signaling in these cells. Meanwhile, DAPT induced a significant reduction of CD44 (5.6-fold) and Slug (several-fold) in the ICN1 cells compared to their untreated counterparts (Fig. 5D; numerical data insert).

CD44 IS REGULATED BY JAGGED-1 VIA NOTCH FAMILY MEMBERS OTHER THAN NOTCH-1

Our data showed that the increased expression of Jagged-1 and Smad-3 induced by constitutively active Notch-1 was not highly affected by DAPT treatment. Meanwhile, Slug and CD44 were greatly reduced indicating a potential role of other Notch family members in regulating stemness and EMT. To further explore findings from the experiments using retrovirally introduced ICN1, parental HCT-116 cells were cultured in the presence of purified Notch ligands followed by investigation of their biological and biochemical properties. Cells were cultured in Jagged-1 coated plates in the presence or absence of DAPT and expression of CD44 was determined. We found that in the presence of Jagged-1, CD44 was increased by 26% compared to cells cultured in the absence of the ligand ($P < 0.014$) (Fig. 6A and

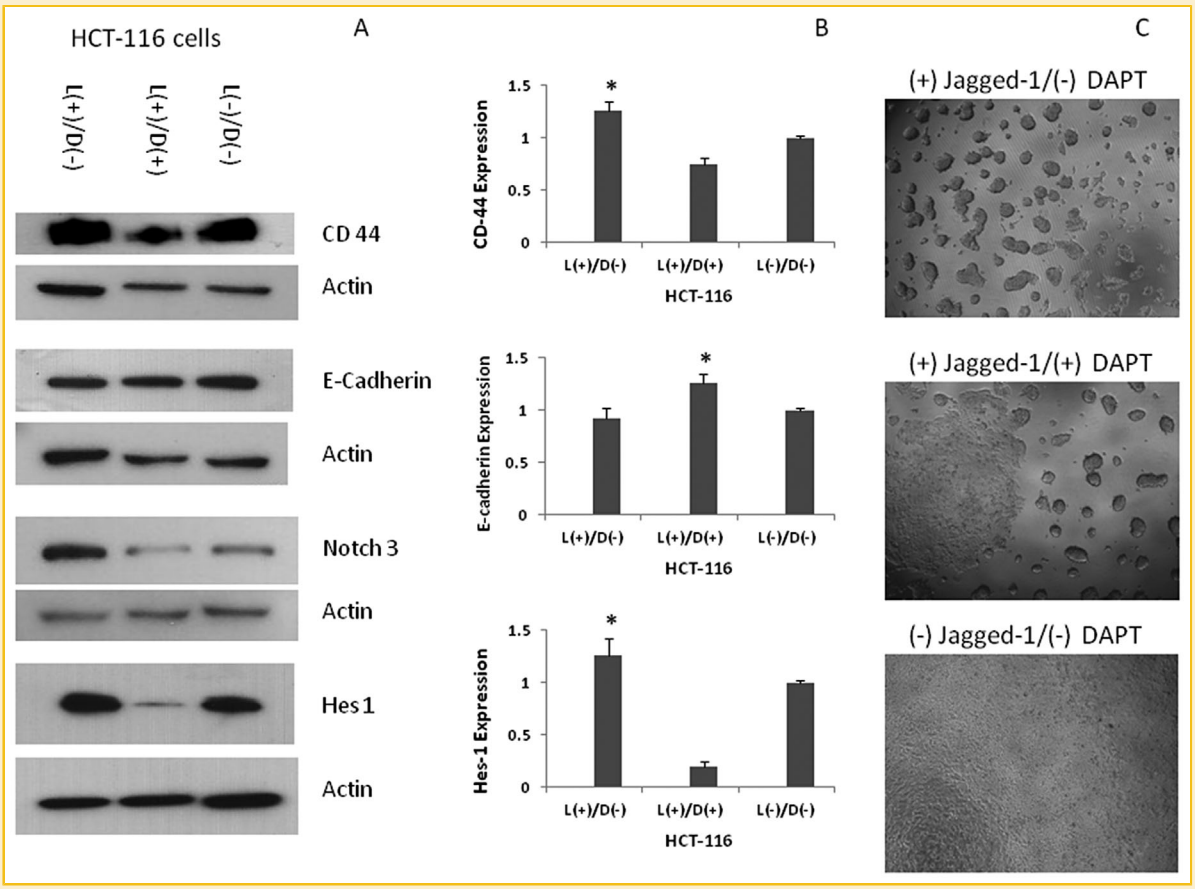

Fig. 6. Jagged-1 ligand upregulates CD44 in colorectal cancer cells. Panels A and B show the expression of CD44, Notch-3, Hes-1, and E-cadherin in HCT-116 cells cultured either in the presence or absence of Jagged-1 and treated with DAPT. Phenotypic alterations of HCT-116 cells plated in Jagged-1 coated plates for 24 h in the presence or absence of DAPT are shown in Panel C. Bars represent means ± SE of protein expression relative to β-actin. *P-value < 0.05. N = 4. L(+), with ligand; L(−), without ligand; D(+), with DAPT; D(−), without DAPT.

B). Meanwhile, DAPT treatment induced a 41% decrease of CD44 in cells cultured in the presence of the Jagged-1 ligand ($P < 0.001$) (Fig. 6A and B). In addition, Jagged-1 induced a 30% increase of the Notch 3 receptor in HCT-116 parental colon cells (Fig. 6A). However, when Jagged-1 exposed cells were treated with the γ-secretase inhibitor, Notch 3 expression was downregulated by 63% (Fig. 6A). DAPT treatment also induced a downregulation of the expression of Hes-1 (Fig. 6A and B). On the other hand, DAPT treatment of HCT-116 grown in the presence of Jagged-1 resulted in a 34% increase of E-cadherin compared to the untreated group ($P < 0.02$) (Fig. 6A and B). Morphological analysis of cells cultured in plates coated with the Jagged-1 ligand showed that the colon cancer cells formed mostly colosphere-like colonies, indicating a more aggressive phenotype (Fig. 6C). This phenotype was partially reversed upon the addition of DAPT in the cultures (Fig. 6C).

DISCUSSION

Although colorectal cancer is highly prevalent in the US, the main cause of death is metastasis to distant organs and the development of secondary tumors [Magredi et al., 2006]. Previous research has indicated that Notch is involved in maintaining cancer-initiating cells in the colon and the inhibition of Notch may prove beneficial in the treatment of CRC [Meng et al., 2010; Sikandar et al., 2010]. In this present work, we report increased Notch-1 expression in colon tumor tissue of CRC patients as compared with patient matched normal colon tissue. Although our patient sample size was limited, our findings are consistent with previous reports [Meng et al., 2010; Sikeandar et al., 2010]. We have extended these findings by showing that expression of constitutively active Notch-1 in the tumor cell line HCT-116 resulted in the induction of EMT/stemness associated proteins and biologic properties often associated with more aggressive colon tumors (e.g., increased transwell migration and anchorage independent growth). Thus, our present work points to a specific biological mechanism that accounts for Notch-mediated effects in colon tumors.

In this paper, the parental HCT-116 cell line was more proficient in repairing wounds than the ICN1 cell line by 1.5-fold. Furthermore, in the present study, colon cells expressing a constitutively active Notch-1 had a 2.5-fold increase in migration and anchorage-

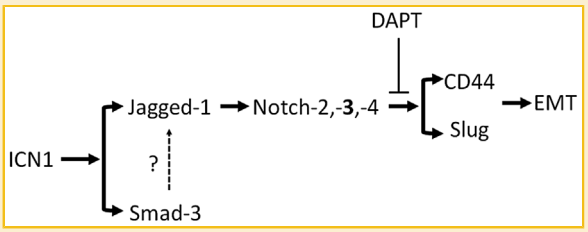

Fig. 7. Model of activated Notch-1 mediated induction of EMT. In our system, constitutively active Notch-1 (ICN1) resulted in increased Jagged-1, Smad-3, CD44, and Slug expression, which is accompanied by acquisition of an EMT-like phenotype in ICN1 expressing cells. Treatment of these cells with DAPT, which inhibits Notch cleavage and thus signaling, resulted in loss of the transfected ICN1 mediated increases in CD44 and Slug. Because transfected ICN1 does not need to be cleaved to signal, and is thus resistant to DAPT treatment, this suggests that ICN1 (Notch-1 signal) upregulates Jagged-1, which in turn activates another Notch-receptor species such as Notch-3 (that are DAPT sensitive), leading to increased CD44 and Slug expression, ultimately resulting in EMT. Increased Smad-3 levels did not change upon DAPT treatment, and are therefore likely directly induced by ICN1. The precise role of Smad-3 (dashed arrows) in the proposed pathway is not addressed by our present study and is part of an ongoing investigation. However, there are reports that Smad can participate in regulating Jagged-1 expression [Ntziachristos et al., 2014].

independent growth over the parental cell line indicating a metastatic and more aggressive phenotype. Notch has previously been identified as being involved in epithelial to mesenchymal transition which is characterized by the transition of cells to a more invasive and mesenchymal phenotype [Sikandar et al., 2010; Garside et al., 2013]. In addition, when cells undergo EMT, they express anchorage-independent growth and obtain the ability to travel throughout the body which ultimately leads to metastasis [Iwatsuki et al., 2010].

The expression of CD44 and Slug was increased in the cells which had constitutively active Notch-1. It has been shown that both proteins are involved in stemness and cancer initiation with CD44 being a stem cell marker and Slug being observed in several stem-cell niches including hematopoietic cells [Yang and Weinberg, 2008; Guadamillas et al., 2011]. Our observation of a decrease in E-Cadherin protein levels is supported by previous findings which have shown a slug-induced repression of E-Cadherin during epithelial-to-mesenchymal transition [Derynck and Zhang, 2003]. HCT-116 cells grown in the wells coated with Jagged-1, a ligand mediating Notch signaling [Iso et al., 2003], had significantly higher CD44 levels compared to the controls, while these cells treated with DAPT and grown in wells coated with Jagged-1 had a 41% decrease in CD44. These results further indicate a more aggressive phenotype and EMT properties associated with the activation of Notch1. Additionally, formation of colosphere-like structure by HCT-116 cells cultured in wells coated with Jagged-1 ligand suggests a more invasive phenotype in cells expressing Notch-1.

Transduction of HCT-116 colon tumor cells with constitutively active Notch-1 resulted in increased transwell migration, increased colony formation in soft agar, but decreased doubling time and wound healing capacity. In addition, the EMT and stemness associated genes, Smad-3, Jagged-1, CD44 and Slug were increased in the presence of ICN1. Treatment of HCT-116 cells expressing ICN1 with DAPT resulted in the retention of Smad-3 and Jagged-1 expression, but a decrease in CD44 and Slug. We interpreted this to indicate that Smad-3 and Jagged-1 expression is directly induced by constitutively active Notch -1 (ICN1), but that CD44 and Slug are induced by other Notch-family members, perhaps through the newly expressed Jagged-1.

A model illustrating this potential pathway is presented in Figure 7. Herein, ICN1 directly induced Smad-3 and Jagged-1 expression. In turn, ICN1 induced Jagged-1 expression results in activation of other Notch-family members such as Notch 3 receptor that results in CD44 and Slug expression, and ultimately EMT. The second step, induction of CD44 and Slug by Jagged-1, is inhibited by DAPT in our system, likely indicating the participation of other Notch-receptors such as Notch 3 in inducing CD44 and Slug expression.

The role for Smad-3 in this pathway is not yet fully known. There are reports that Smads, via TGF-β signaling, can result in Jagged-1 expression in tumor metastasis [Ntziachristos et al., 2014] Examination of TGF-β receptor expression in our system yielded inconclusive results, with TGF- β-R being induced by ICN1 in some experiments, but no change in other experiments (data not shown). However, Smad can also be activated by other pathways such as BMP and WNT, which have also been reported to intersect with Notch, adding another layer of complexity (reviewed in [Bertrand et al., 2012]).

Alterations in Notch-1 or alteration in genes that regulate Notch, resulting in constitutive activation, have been reported in colon cancer [Reedijk et al., 2008; Meng et al., 2009; Miyamoto and Rosenberg, 2011]. Moreover, increased expression of stemness and EMT markers has been reported to be associated with colon cancer recurrence and a poor prognosis [Scheel and Weinberg, 2012; Vaiopoulos et al., 2012]. Our data demonstrate that constitutively active Notch-1 can induce EMT and stemness markers through a pathway that likely involves multiple Notch receptors, and as such provides novel biological insight into the rational for the therapeutic potential of Notch inhibitors in colon cancer.

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