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Upregulated JAG1 Enhances Cell Proliferation in Adrenocortical Carcinoma

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Abstract

Purpose—The purpose of this study was to examine the expression and molecular significance of JAG1, a ligand for the Notch developmental signaling pathway, in adrenocortical carcinoma (ACC).

Experimental Design—Human microarray data were analyzed for genes expressing ligands for the Notch pathway and validated with QPCR and immunoblots of RNA and protein, respectively. ACC cell lines were assessed for Notch pathway member expression by immunoblot, QPCR, and immunofluorescence. Notch pathway activity was also determined utilizing a reporter gene (luciferase) activation. Proliferation experiments employing a Jag1 knockdown strategy (Jag1KD) and a inhibitor of Notch-dependent transcription (DNMaml), utilized a co-culture system with FACS analysis. Tumor stage and mitotic rate of human ACC samples were correlated to JAG1 expression.

Results—The Notch ligand JAG1 mRNA and protein are upregulated in ACC. JAG1 upregulation can be modeled in the Y1 mouse ACC cell line that expresses Jag1, Notch receptors, downstream signaling molecules, and exhibits density-dependent Notch activation. Jag1 enhances cell proliferation through activation of canonical Notch signaling as shown through Jag1 knockdown (Jag1KD) and co-culture experiments. Inhibition of Notch signaling at the level of post receptor signaling (DNMaml), results in similar inhibition of cell proliferation. Analysis of clinical data indicates Jag1 expression correlates with both Grade and Stage of ACC supporting a role of JAG1-dependent Notch activation in late-stage ACC.

Conclusions—JAG1 is the primary upregulated Notch ligand in ACC and enhances ACC cell proliferation and tumor aggressiveness in a non-cell-autonomous manner through activation of Notch signaling in adjacent cells.

Keywords

JAG1; adrenocortical carcinoma; Notch signaling; non-cellautonomous

Introduction

Adrenocortical Tumors (ACT) are extremely common neoplasms, the vast majority being benign adrenocortical adenomas (ACA) that occur in as many as 4–7% of the population whereas adrenocortical carcinomas (ACC) are extremely rare (~0.5–2 cases/million) accounting for 0.2% of cancer deaths annually (1, 2). ACC is typically an aggressive neoplasm with many patients presenting with metastases upon diagnosis (1). Due to difficulty of early detection and lack of effective treatments for advanced-stage ACC, the average survival for surgically unresectable tumors is 12-months and the overall 5 year survival is historically less than 10% (3, 4). The molecular pathogenesis of ACC has remained elusive until recently. Dysregulation of developmental signal transduction pathways is found in an increasing number of cancers including ACC. Specifically, the Wnt signaling pathway, a critical mediator of adrenal development, plays an important role in the etiology of ACC, where constitutively active, nuclear β -catenin is frequently observed (5–9). The development of visible adrenal tumors in mice engineered to express constitutively-active β -catenin in the mouse adrenal cortex supports the hypothesis that dysregulation of Wnt/ β -catenin signaling is a vital step in adrenocortical tumorigenesis (10).

Similar to the Wnt pathway, Notch signaling is involved in a wide range of cell fate decisions during development. While its dysregulation is a common molecular event in a variety of cancers, its role in adrenal development and ACC is unknown (11, 12). Notch signaling involves interaction between a transmembrane ligand, of either the Jagged (JAG1/2) or Delta-like (DLL1/3/4) family, and a transmembrane receptor (NOTCH1/2/3/4) generally expressed on adjacent cells (13, 14). Upon binding of Notch ligand to receptor, the γ -secretase complex cleaves the Notch receptor in two locations releasing the active signaling molecule NICD (cleaved Notch intercellular domain). NICD interacts with constitutively DNA-bound CSL (CBF-1/RBPjk/Su(H)/Lag-1), recruits the essential transcriptional coactivator MAML (Mastermind-like) and initiates transcription of Notch-dependent genes such as the HES (hairly enhancer of split) family of transcription factors.

The upregulation of the Notch ligand, Jagged1 (JAG1), in a variety of cancers implies a ligand-dependent activation of the Notch signaling pathway (15–17). Indeed, the upregulation of JAG1 in breast and prostate cancer has been implicated in metastatic disease and correlated with poor prognosis (18–21). Mechanistically, JAG1 is thought to enhance the metastatic potential of breast cancer through a Notch-dependent induction of epithelial-to-mesenchymal transition of mammary epithelial cells (22). Like all Notch ligands, JAG1 classically interacts with receptors on adjacent cells (non-cell-autonomous) rather than with receptors on the cells in which they are expressed (cell-autonomous). However, the ability of JAG1 to induce malignant transformation of RKE cells despite the absence of Notch receptors raises the possibility that non-canonical actions of JAG1 mediate some of its oncogenic manifestations (23). In this study, we report for the first time that JAG1 is the primary upregulated Notch ligand in ACC and enhances ACC cell proliferation and tumor aggressiveness in a non-cell-autonomous manner through activation of Notch signaling in adjacent cells.

Materials and Methods

Microarray Analysis

DNA Microarray analyses were performed with Affymetrix U133A 2.0 Plus oligonucleotide arrays and have been published (24, 25). Probe sets for JAG1, JAG2, DLL1, DLL3, and DLL4 were presented in a heatmap with clustering delineated by tumor type; individual samples were ordered based on JAG1 expression as determined by probe sets 216268_s_at and 209099_x_at. Data presented as dot plots used the following probe sets: JAG1 Set #1

231183_s_at, JAG1 Set #2 209099_x_at, JAG2 Set #1 209784_s_at, JAG2 Set #2 32137_at. Correlations were performed using JAG1 probe set 216268_s_at with KI67 212022_s_at, and with TOP2A 201292_at. Similar correlations were obtained with other JAG1 probe sets.

Human Samples

Protein and RNA were extracted using routine protocols from frozen adrenocortical tissues obtained via the University of Michigan Comprehensive Cancer Center Tissue Procurement Service with IRB approval. Samples for protein and RNA analysis were randomly selected [normal adrenal (NL): n=5, ACA: n=5, ACC: n=10]. Due to tissue availability, different pools of samples were analyzed for message and protein.

Plasmids, shRNA and transfection

Notch reporter (pJH23A: 4xwtCBF1Luc) and Control Reporter (pJH25A: 4xmtCBF1Luc) expression vectors were a generous gift from Dr. S. Dianne Hayward (John Hopkins University Medical School, Baltimore, MD) (26). The Notch reporter contains four consensus CSL binding sites driving expression of firefly luciferase while these sites are mutated in the Control Reporter. pGIPZ vectors (Open Biosystems, Huntsville, AL) expressing shRNA against JAG1 and a non-specific scrambled control shRNA (Scramble) were obtained from the University of Michigan shRNA core (<http://fgc.lsi.umich.edu/index.html>). In addition to the shRNA, pGIPZ vectors contain a puromycin selection cassette and an IRES GFP sequence. Sequences for JAG1 shRNA are #1: 5'-gtcagaattgtgacataaa-3' and #2: 5-gggatttggttaatggtta-3'. pdsREDII expresses dsREDII under control of the CMV promoter and was obtained from Dr. Claudius Vincenz (University of Michigan, Ann Arbor, MI). Control (MigR1) plasmid, which expresses GFP, and DNMAm1 plasmid, which expresses a fusion protein of GFP and amino acids 13–74 of Maml1 and acts as a dominant-negative, were a generous gift of Dr. Ivan Maillard (University of Michigan) (27, 28). Both Control (MigR1) and DNMAm1 plasmids contain flanking LTR sequences and expression is driven by an MSCV promoter. Retroviral packaging protein expression plasmids pGag/Pol and pVSV were kindly provided by Dr. Michael Malim (King's College, London, UK).

Cell Culture and Generation of Stable Cell Lines

Culture of the mouse ACC cell line Y1 (29) and the Human ACC cell lines NCI-H295A (30) and RL251 (31) has been described previously (25, 32). All standard cell culture reagents were obtained from Invitrogen Life Technologies (Carlsbad, CA). Virus-competent 293T cells, a gift from Dr. Benjamin Margolis (University of Michigan), were maintained in DMEM with 10% Cosmic Calf Serum (CCS, Hyclone, Logan, UT) and penicillin/streptomycin. In some experiments, Y1 cells were treated for 6h with 5mM EDTA prepared in PBS. Transient transfections were performed using Eugene (Roche, Madison, WI) according to the manufacturer's instructions and optimized at a 4:1 ratio (4 µl Eugene/1 µg DNA) for Y1 cells and 2:1 ratio for 293T cells.

For generation of Scramble (GFP+) and Jag1KD (GFP+) stable cell lines, Y1 cells were transfected with 2 µg of pGIPZ vectors expressing shRNA directed against Jag1 or a control (Scramble) as described above, followed by 4 weeks of puromycin selection (2 µg/ml, Roche). Cells were then enriched for GFP expression within the 10⁴–10⁵ range using Fluorescence Activated Cell Sorting (FACS) as described below. Sorted cells were replated in 10cm dishes and allowed to expand.

To obtain the dsREDII [wildtype Y1 (Red+)] cell line, 10cm dishes of Y1 cells were transiently transfected with 2 µg of pdsREDII as described. Because pdsREDII lacks a mammalian selection cassette, cells were passaged after 2 days and were transiently

transfected an additional time. After 2 days cells were enriched for dsREDII expression within the 10^4 – 10^5 range by FACS. Sorted cells were replated in 10cm dishes and allowed to expand.

To generate the Control (GFP+) and DNMaml (GFP+) stable cell lines, viral supernatant was generated by cotransfection of 293T cells with 2 μ g each of pGag/Pol, pVSV, and either Control (MigR1) or DNMaml constructs. After 2 days, medium was collected and centrifuged at 5,000 $\times$ g followed by filtration through a 0.22 μ m syringe nylon filter (Fisher Scientific, Pittsburgh, PA). Viral supernatant was adjusted to a final volume of 10ml with DMEM and polybrene was added (10U/ml, Sigma, St. Louis, MO). Y1 cells were then transduced with viral supernatant for 24 hours. Cells were passaged and transduced an additional time under identical conditions. Cells were then enriched for GFP expression within 10^4 – 10^5 range using FACS and were replated into 10cm dishes and allowed to expand. Because the GFP and dsREDII expression evidently diminished overtime, cells were resorted under identical parameters every 3 months.

Fluorescence Activated Cell Sorting and Analysis

Trypsinized cells were pelleted at 1,000 $\times$ g for 5min and resuspended in 1 $\times$ PBS containing 10% CCS at a concentration of 1–2million cells/ml. FACS experiments were performed by the University of Michigan Flow Cytometry Core (<http://www.med.umich.edu/flowcytometry/>) with either BD Biosciences FACSDiVa High-Speed Cell Sorter (3-laser: 488nm, 350nm and 633nm) or BD Biosciences FACSARIA High-Speed Cell Sorter (3-laser: 488nm, 407nm and 633nm).

Quantitative real time-PCR (QPCR) Analysis

RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA) according the manufacturer's instructions and cDNA was generated using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). QPCR experiments were performed as previously described (33, 34). A comprehensive list of human and mouse QPCR primers is found in Supplementary Table 1. Analysis was conducted with either the efficiency-corrected Δ CT method or the $\Delta\Delta$ CT method as indicated (35). Expression of mRNA was normalized to β -actin.

Immunocytochemistry and Immunoblots

For a comprehensive list of primary and secondary antibodies used for Immunocytochemistry (ICC) and Immunoblots see Supplementary Table 2. For immunocytochemical localization: Y1 cells were plated on glass slides coated with fibronectin (10 μ g/ml, Sigma). Slides were washed in 1 $\times$ PBS, fixed in 4% paraformaldehyde (Fisher) for 15min at 4°C, and permeabilized with 0.02% Igepal CA-630 (Sigma). Slides were blocked with 2% milk in 1 $\times$ PBS and primary/secondary antibodies (Supplementary Table 2) were diluted in 0.2% milk in 1 $\times$ PBS. For detection of native fluorescence, slides were not fixed in order to preserve the activity of GFP and dsRedII. Cover slips were applied and images obtained as previously described (33, 34).

Immunoblot analysis of protein lysates from cell cultures were performed as previously described (33). Analysis of some protein lysate was conducted as described but blocking, primary, and secondary dilutions were done in Odyssey Blocking buffer (LICOR, Lincoln, NE), secondary antibodies used were Odyssey IRdyes (Supplementary Table 2). Immunoblots using protein lysate from human adrenal tumor samples was quantified using ImageJ software (National Institutes of Health, Bethesda, Md). The btan20 (Notch1) and C651.6DbHN (Notch2) monoclonal antibodies were developed by Spyros Artavanis-Tsakonas and were obtained from the Developmental Studies Hybridoma Bank under the

auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, 52242.

MTS Proliferation Assay

The MTS cell viability assay translates cell number into a colorimetric readout (absorbance) via metabolic breakdown of tetrazolium salts (Promega Corp, Madison, WI). Cells were plated in 96-well plates and assay performed according to the manufacturer's instructions. Absorbance values were obtained using a SpectraMAX190 plate reader (Molecular Devices, Sunnyvale, CA).

Co-culture Experiments

Stable cell lines of Y1 cells expressing dsRedII [wildtype Y1 (Red+)] were co-cultured with either Scramble (GFP+) or Jag1KD (GFP+) cells in triplicate wells of a 6-well plate in two ratios: 90% Red+/10% GFP+ and 10% Red+/90% GFP+. The combined initial concentration for each ratio at each time point was 150,000 cells/well. Cells analyzed at the Day 4 time point were plated 4 days in advance of analysis, Day 3 time point were plated 3 days in advance of analysis, etc. At the end of the 4-day time course, harvested cells were analyzed by FACS as described above. 10,000 cells were analyzed for each sample and the cell number for each color (Red+ or GFP+) was determined and data are presented as a percentage change from Day 1 (For schematic, Fig 4A). Identical conditions and analysis were performed for co-culture of wildtype Y1 (Red+) with either Control (GFP+) or DN Maml (GFP+) except that a 50%/50% ratio was used.

Luciferase Assays

Y1 cells were plated in 24-well plates and were transiently transfected with 50ng of pRL-TK *Renilla* Luciferase (Promega Corp, Madison, WI) and 0.5 µg of either Control Reporter (pJH25A) or Notch Reporter (pJH23A) firefly luciferase constructs described above. Assays were performed 24h after transfection using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturers instructions and optimized for Y1 cells. Cells were lysed in 1× Passive Lysis Buffer and lysates analyzed on the Glomax Multi-detection System (Promega). Expression was normalized to pRL-TK *Renilla* Luciferase.

Statistics

All comparisons made used the student's T-Test. Statistical analysis of microarrays has been described elsewhere (24, 36).

Results

JAG1 is Upregulated in Human ACC

In an effort to better understand the molecular characteristics of human ACC, our group has previously performed DNA microarray analyses using frozen human tissues – most recently with a total of 33 ACC, 22 ACA, and 10 normal adrenals (NL) (24, 36). Analysis of differentially-expressed probe sets revealed an upregulation of the Notch ligand JAG1 in ACC samples compared to normal and adenomatous tissue (Fig 1A). The five JAG1 probes sets depicted are within the top 0.8% of all rank-ordered upregulated probe sets represented in the microarray. The other four Notch ligands (JAG2, DLL1/3/4) were upregulated in only a few ACC.

Quantitative analysis of two independent probe sets for each JAG ligand (JAG1 and JAG2) confirmed that JAG1 expression is significantly higher in ACC samples compared to NL and ACA (Top panels, Fig 1B). JAG2 exhibits a statistically significant, albeit less dramatic,

difference in expression among samples (Bottom panels, Fig 1B). Furthermore, interrogating two other adrenal tumor microarray data sets revealed a similar upregulation of JAG1 in ACC (36, 37).

Microarray analyses were validated with QPCR of mRNA from human adrenal tumor samples (Fig 1C). Correlation of QPCR and microarray data for each samples is shown in Fig 1D. While both JAG1 and JAG2 were significantly different in ACC vs ACA/NL, JAG1 is expressed at a higher level and a greater increase in ACC than JAG2. Additionally, JAG1 QPCR expression was more tightly correlated to the microarray data (JAG1: $r=0.874$, JAG2= 0.545). These data support the validity and biological relevance of the microarray results.

Furthermore, immunoblot analysis of human adrenal tumor samples revealed a higher expression of JAG1 protein in the majority of ACC when compared to NL and ACA (Top panel, Fig 1E). Quantification of band intensity of two immunoblots using the same set of human samples identifies robust protein levels of JAG1 in most ACC and barely detectable quantities in ACA and NL (Bottom panel, Fig 1E). Together these data suggest that JAG1 mRNA and protein is upregulated in a majority of ACC samples and is consistent with biological relevance of JAG1-activated Notch signaling contributing to adrenocortical carcinogenesis. While JAG2 is also upregulated, it exhibits a lower level of expression and a poorer correlation of QPCR and array data. Therefore, we decided to focus exclusively on JAG1, the significance of its upregulation in ACC, and its role in adrenocortical carcinogenesis.

The Y1 Mouse ACC Cell Line Exhibits Active Notch Signaling

Predicated on the canonical role of JAG1 as an obligate ligand for Notch activation, normal mouse adrenal (Adrenal), the mouse ACC cell line (Y1), and the two cell lines derived from human ACC (H295A and RL251) were surveyed for concurrent Jag1 abundance and expression of the Notch signaling components. Jag1 protein is highly expressed in both Y1 and H295A lines, recapitulating in an *in vitro* context the upregulation of JAG1 observed in the human ACC samples (Fig 2A), with Jag1 mRNA showing a 43-fold increase over Jag2 and Dll1/3/4 ligands being barely detectable (Fig 2B). This latter comparison demonstrates that the Y1 cell recapitulates the Notch ligand expression profile observed in the human ACC microarray where the expression of the other four Notch ligands is only modestly elevated and is consistent with Jag1 functioning as the biologically relevant Notch ligand in Y1 cells. Y1 cells also express both the Notch1 and Notch2 receptors together with the active signaling molecule NICD. Immunocytochemistry (ICC) reveals ubiquitous expression of core Notch pathway components (ligand: Jagged1, receptors: Notch1/2, target gene: Hes1) in Y1 cells (Fig 2C), suggesting that juxtaposed Y1 cells are capable of activating canonical Notch signaling in adjacent cells and/or are capable of self-activation (ie: ligand-mediated receptor activation).

The active engagement of Notch signaling in Y1 cells provides an appropriate model system to examine Jag1-dependent Notch activation in ACC. Because Mg^{2+} is required for Notch receptor stability, Mg^{2+} depletion can be used to induce Notch receptor cleavage and biochemically release the active NICD peptide in order to test induction of Notch-dependent transcription (as opposed to ligand-independent constitutively active Notch-mediated transcription) in Jag1-expressing tumor cells (38, 39). Treatment of Y1 cells for 6 hours with the chelator EDTA to deplete Mg^{2+} resulted in an increase in NICD protein in EDTA treated cells versus vehicle treated cells (Top panel, Fig 2D). H295A human ACC cells also exhibited a similar response to EDTA treatment (Supplementary Fig 1). To confirm NICD cleavage results in productive Notch-dependent transduction, Y1 cells were transiently transfected with a specific Notch luciferase reporter containing four NICD-consensus

binding sites or an otherwise identical reporter in which the NICD sites are mutated. Following EDTA treatment, an increase in the expression of the Notch luciferase reporter was observed (EDTA-Notch versus Vehicle-Notch = 2.3 fold increase, bottom panel, Fig 2D). These data indicate canonical Notch signaling can be activated in Y1 cells, presumably due to the presence of Jag1.

Because Notch signaling is generally dependent upon the juxtaposition of two adjacent cells expressing membrane-bound ligand and receptor respectively, we hypothesized that activation of Notch signaling in Y1 cells is density dependent. When Y1 cells were plated at increasing density (10%, 25%, 50%, 90% confluence) a density-dependent increase in NICD protein was observed (Top panel, Fig 2E). When the transcriptional activity of the Notch reporter was evaluated by luciferase assay, an elevated activity was observed in the highest (90%) when compared to the lowest (10%) density (High Density-Notch versus Low Density-Notch = 2.3 fold, bottom panel, Fig 2E). Additionally, immunoblot analysis did not detect Jag1 in conditioned medium from Y1 cells, which eliminates the possibility that Jag1 is acting as a secreted factor (data not shown). Taken together, these data confirm active Notch signaling is occurring in Y1 cells.

To investigate the dependence of Notch activation on Jag1 in ACC cells, a shRNA knockdown strategy was employed. Two shRNAs with 100% homology to Jag1 mRNA were found to be sufficient to knockdown Jag1 in Y1 cells when used in combination. Two stable cell lines were generated, a Jag1-knockdown line expressing both Jag1 shRNAs [Jag1KD (GFP+)] and a control line expressing a non-specific shRNA [Scramble (GFP+)]. GFP is expressed concurrently with the shRNA and scramble vectors; thus both cell lines are GFP+. Jag1 protein was significantly decreased in the Jag1KD (GFP+) cell line, an effect that was stable for more than three weeks as determined by immunoblot analysis (Fig 3A). Jag1 mRNA expression was also reduced by 63% while the related ligand Jag2 showed no statistically significant change (Fig 3B). The concurrent suppression of the Notch target gene *Hes1* is consistent with a Jag1-dependent activation of Notch signaling in ACC cells.

Jag1 has a Non-cell-autonomous Effect on ACC Cell Proliferation

To analyze the effect of Jag1 knockdown on proliferation in Y1 cells, the MTS viability assay was utilized. While no difference in proliferation was observed between Scramble (GFP+) and Jag1KD (GFP+) cells when plated at low density (10% confluence, Fig 3C), Jag1KD (GFP+) cells showed a 32% reduction in proliferation when plated at a higher concentration (40% confluence, Fig 3D). These data confirm that Jag1 exerts an effect on ACC cell proliferation in a density-dependent manner. Furthermore, immunoblot analysis revealed a reduction in Proliferating Cell Nuclear Antigen (PCNA), a marker of proliferation, in the Jag1KD (GFP+) cell lysates compared to Scramble (GFP+), while Cleaved-Caspase-3 (Civ-Csp-3), a marker of apoptosis, was not detected in either cell line (Supplementary Figure 2A and data not shown). These data confirm that a loss of Jag1 protein inhibits the proliferation of Y1 cells. Predicated on the assumption that Jag1 acts through Notch receptors on adjacent cells, it would be expected to influence proliferation in a non-cell-autonomous manner consistent with the density dependence observed.

In light of recent data that suggest additional Notch receptor-independent biological functions of Jag1 and the related Notch ligands DLL1 and DLL3 within the cells in which they are expressed (cell-autonomous) (23, 40, 41), a co-culture system was designed utilizing FACS analysis to further interrogate the hypothesis that Jag1 acts in a non-cell-autonomous manner in ACC cells. Jag1KD (GFP+) or Scramble (GFP+) Y1 cell lines were grown in combination with wildtype Y1 cells expressing dsRedII [wildtype Y1 (Red+)] (Figure 4A). Wildtype Y1 (Red+) cells were cultured with Scramble (GFP+) or Jag1KD (GFP+) in two different ratios: 90% Red+/10% GFP+ or 10% Red+/90% GFP+ (Figure 4B).

The former condition (90% Red+/10% GFP+) assessed the ability of Jag1KD (GFP+) [relative to the Scramble (GFP+)] cells to proliferate upon receiving Jag1 inputs from wildtype Y1 (Red+) while the latter condition (10% Red+/90% GFP+) interrogates the effect of decreased Jag1 input to wildtype Y1 (Red+) cells. To guarantee sufficient cellular interactions, cells were initially plated at high density (70% confluence by Day 2, Fig 4B). To assure all cells could be analyzed at the same time at the end of the four day time course, the same initial concentration of cells were plated four days prior to analysis for Day 4, three days prior to analysis for Day 3, etc (Fig 4A). Cell number for each FACS-sorted Red+ and GFP+ populations was determined at each time point and relative proliferation of the populations is presented as a percentage change in these cell numbers from Day 1 (Fig 4A).

To examine the hypothesis that Jag1 functions in a non-cell-autonomous manner, the following mixing experiment was performed. In the 90% Red+/10% GFP+ ratio, Jag1KD (GFP+) cells were cultured with an abundance of wildtype Y1 (Red+) cells expressing high amounts of Jag1, thus Jag1KD (GFP+) cells should be able to receive an abundance of Jag1 signaling inputs from neighboring wildtype Y1 (Red+) cells (Left panel, Fig 4B). Under these conditions, wildtype Y1 (Red+) cells show no relative change in proliferation whether cultured with Scramble (GFP+) and Jag1KD (GFP+) cells. This is expected since wildtype Y1 (Red+) cells are most likely receiving Jag1 inputs predominantly from other wildtype Y1 (Red+) cells (Top panel, Fig 4C, Supplementary Fig 3). Importantly, no difference in the proliferation of Scramble (GFP+) and Jag1KD (GFP+) cells are observed under these co-culture conditions (Bottom panel, Fig 4C, Supplementary Fig 3). These data indicate that the Scramble (GFP+) and Jag1KD (GFP+) cells are capable of receiving Jag1 inputs from wildtype Y1 (Red+) cells and, hence, proliferate normally supporting the hypothesis that Jag1 has a non-cell-autonomous effect (Supplementary Fig 3).

To further test the hypothesis that Jag1 acts in a non-cell-autonomous manner on adjacent cells, wildtype Y1 (Red+) cells were cultured with Jag1KD (GFP+) or Scramble (GFP+) cells at the ratio of 10% Red+/90% GFP+ (Right panel, Fig 4B). Under these conditions, wildtype Y1 (Red+) cells receive a majority of signaling input from GFP+ (Scramble or Jag1KD) cells. Specifically, the wildtype Y1 (Red+) cells receive numerous Jag1 inputs from Scramble (GFP+) cells and reduced Jag1 inputs from the Jag1KD (GFP+) cells (Right panel, Fig 4B, Supplementary Figure 3). Under these conditions, wildtype Y1 (Red+) cells show a 23% reduction in proliferation at Day 2 and a 27% reduction in proliferation at Day 3 (when co-cultured with Jag1KD (GFP+) cells (Top panel, Fig 4D). The Jag1KD (GFP+) cells also exhibit a maximal 35% reduction at Day 4 (Bottom panel, Fig 4D) predicated on the assumption that they are receiving the majority of signaling inputs from neighboring Jag1KD (GFP+) cells.

In summary, wildtype Y1 (Red+) cells proliferate less well when co-cultured with 90% Jag1KD (GFP+) cells suggesting that a decrease of Jag1 inputs results in retarded Y1 cell growth. Jag1KD (GFP+) cells remain competent to receive Jag1 inputs from wildtype Y1 (Red+) cells as reflected in the increased proliferation of the Jag1KD (GFP+) cells grown in the presence of 90% wildtype Y1 (Red+) cells. Jag1KD (GFP+) cells proliferated less well in the co-culture containing 90% Jag1KD (GFP+) cells suggesting that the decrease in Jag1 inputs results in retarded Jag1KD (GFP+) cell growth. Together, the co-culture studies indicate that Jag1 enhances ACC cell proliferation in a non-cell-autonomous manner. See experimental model in Supplementary Figure 3.

Inhibition of Notch-Dependent Transcription Reduces ACC Cell Proliferation

The non-cell autonomous enhancement of ACC cell proliferation by Jag1 is consistent with a Notch receptor-dependent process. As such, an inhibition of Notch-dependent transcription should phenocopy the Jag1 knockdown in a cell-autonomous manner. Notch-dependent

transcription is initiated by a ternary complex of the basally-repressive CSL, active signaling molecule NICD, and transcriptional coactivator MAML (1–4) (13, 14). An engineered peptide sequence derived from Maml1, which has a dominant-negative effect on all Notch-dependent transcription by competing for the endogenous Maml proteins and preventing their binding to NICD and CSL (27, 28) was utilized [DNMaml (GFP+): expresses GFP fusion protein of amino acids 13–74 of Maml1, Control (GFP+): expresses GFP]. Stable cell lines expressing either DNMaml or the Control construct were generated and RNA was isolated and analyzed by QPCR. In DNMaml (GFP+) cells, the canonical Notch target gene *Hes1* and the putative target *Cdkn1a* are reduced by 64% and 43% respectively (Fig 5A). Two unrelated but highly expressed genes in Y1 cells, *Ctnnb1* (β -catenin) and *Sf1* (steroidogenic factor 1) were unaffected consistent with a specific inhibition of Notch target genes in the DNMaml (GFP+) cell line.

Since DNMaml inhibits Notch-dependent transcription, we hypothesized that DNMaml (GFP+) cells would have a reduced ability to proliferate when compared to Control (GFP+) cells. Employing the MTS viability assay to assess proliferation, DNMaml (GFP+) cells plated at 40% confluence showed a 37% reduction in proliferation when compared to Control (GFP+) cells (Fig 5B). Furthermore, immunoblot analysis revealed a reduction in PCNA protein level in DNMaml (GFP+) cell lysates compared to Control (GFP+), while Clv-Csp-3 was undetectable and the protein level of Cleaved-Caspase-6 (Clv-Csp-6), another marker of apoptosis, was unchanged (Supplementary Fig 2B).

While Jag1 functions non-cell-autonomously to influence ACC cell proliferation, DNMaml targets downstream Notch signaling and should have a cell-autonomous effect on proliferation. To directly address this supposition, a similar co-culture study was performed using a 50% wildtype Y1 (Red+)/50% Control (GFP+) or DNMaml (GFP+) ratio. Wildtype Y1 (Red+) cells co-cultured with either Control (GFP+) or DNMaml (GFP+) cells maintain robust proliferation (Left panel, Fig 5C). No statistically significant difference in *Hes1* expression was observed on Day 4 in wildtype Y1 (Red+) cells cultured with GFP+ (Control or DNMaml) cells indicating DNMaml is not affecting Notch signaling in adjacent wildtype Y1 (Red+) cells (Right panel, Fig 5C). Conversely, DNMaml (GFP+) cells cultured with wildtype Y1 (Red+) cells exhibit a 34.3% reduction in proliferation when compared to Control (GFP+) cells cultured with wildtype Y1 (Red+) cells (Left panel, Fig 5D). *Hes1* mRNA was reduced 71.34% in DNMaml (GFP+) versus Control (GFP+) cells at Day 4 (Right panel, Fig 5D). These data indicate DNMaml is acting specifically in DNMaml (GFP+) cells. Together with the Jag1 co-culture studies, these data support a Jag1 dependent activation of Notch signaling in ACC that can be targeted at the level of ligand (presenting cell) or receptor (receiving cell) to inhibit ACC cell proliferation.

JAG1 expression is correlated with increased aggressiveness of ACC

JAG1 is upregulated in ACC and acts through canonical Notch signaling to enhance density-dependent ACC cell proliferation. To determine whether elevated JAG1 mRNA expression levels in human ACC correspond to an increase in cancer aggressiveness, tumor stage and grade (as assessed by mitotic rate) were examined in the 33 ACC samples used in the microarray analysis. JAG1 mRNA expression levels correlated with advanced stage ($r=0.35$; $p=0.04$) and with mitotic rate ($r=0.40$; $p=0.02$) (Fig 6A, 6B). Specifically, JAG1 expression was increased 1.67 fold ($p=0.05$) in late stage ACC (Stage III and IV) compared to early stage ACC (Stage I and II) (Fig 6A). Our previous microarray has shown strong correlations between KI67 and Topoisomerase 2A (TOP2A) expression, two markers of proliferation that are highly upregulated in ACC, and immunohistochemical staining for Ki67 and Top2a protein (36). We identified a positive correlation of JAG1 expression with KI67 expression (overall correlation $r=0.62$, $p<0.0001$) and TOP2A (overall correlation $r=0.69$, $p<0.0001$).

These data are consistent with the significant role of JAG1 in ACC cell proliferation and advanced stage of disease.

Discussion

The Notch ligand JAG1 mRNA and protein are upregulated in adrenocortical carcinoma (ACC). JAG1 upregulation can be modeled in the Y1 mouse ACC cell line that expresses Jag1, Notch receptors, and downstream signaling molecules. Y1 cells exhibit density-dependent Notch activation. Jag1 enhances cell proliferation through activation of canonical Notch signaling as shown through knockdown and co-culture experiments. Inhibition of Notch signaling at the level of ligand (Jag1KD) or post receptor signaling (DNMaml), results in similar inhibition of cell proliferation. Analysis of clinical data indicates Jag1 expression correlates with both Grade and Stage of ACC supporting a role of JAG1-dependent Notch activation in ACC.

JAG1 upregulation has been observed in several cancers such as breast and prostate cancer where it facilitates proliferation and metastasis (22, 40). In breast cancer, JAG1 is correlated with poor prognosis and lower survival rates in women with late stage, aggressive cancer (18–20). Mechanistically, JAG1 has been shown to induce expression of cyclinD1 in prostate cancer (21), enhance the number of cancer cells in S-phase (41), and facilitate proliferation in Wnt1-transformed breast epithelial cells (42, 43).

While the canonical mechanism by which Jag1 mediates cellular effects in numerous systems is through its binding to the Notch receptors and activation of downstream signaling (11–14), Jag1 and the other Notch ligands may also have receptor independent roles (23, 44, 45). Overexpression of Jag1 has been shown to cell-autonomously induce transformation of RKE cells independent of Notch receptors but dependent on intracellular interaction between the cytoplasmic tail of Jag1 and Affadin, a cell adherens junction protein (23). Furthermore, Jag1 and DLL1 are able to be processed by the γ -secretase complex to release intracellular signaling fragments (44). In this report, knockdown of Jag1 in mouse adrenocortical cancer cells employing specific shRNAs resulted in a density-dependent reduction in proliferation. Co-culture experiments of normal Y1 cells with either Jag1KD or Scramble cell lines tested whether Jag1 has a cell-autonomous or non-cell-autonomous effect. Jag1KD cells were competent to proliferate provided they received sufficient Jag1 signaling inputs from adjacent cells. Cells receiving diminished Jag1 inputs from Jag1KD cells did not proliferate as well as cells receiving inputs from control (Scramble) cells. These data indicate Jag1 does not have a cell-autonomous effect but instead mediates adrenal cancer cell proliferation by binding to and activating Notch receptors on adjacent cells. The similar cell-autonomous reduction of growth following inhibition of Notch-dependent transcription utilizing a dominant negative version of the transcriptional coactivator Maml1 supports the conclusion that Jag1 effects ACC cell proliferation in a non-cell-autonomous manner.

Of obvious interest is the molecular mechanism of JAG1 upregulation in human ACC. It is informative that Wnt and Notch are known to synergize in a variety of developmental systems such as the ear where Jag1 acts to mediate some of the effects of downstream Wnt/ β -Catenin signaling on the formation of the otic placode (46). Moreover, JAG1 has been shown to be a direct target of β -catenin in the epidermis where Notch signaling is required for β -catenin mediated melanoma formation (47, 48). A synergistic effect between Notch and Wnt on tumorigenesis is also seen in breast and colon carcinoma where JAG1 is upregulated in both of these cancers (15, 18, 42, 49).

Whether the Notch and Wnt pathways interact in ACC is unknown. While repression of Notch-dependent transcription had no effect on β -catenin (Ctnnb1) expression in the DN β Maml experiments (Fig 5A), it remains unknown if Wnt activation synergizes or activates various components of the Notch pathway. Nuclear β -catenin has been observed in both benign ACAs and malignant ACCs (5–9) as well as the known ACC cell lines H295A and Y1 (Kim and Hammer, unpublished observations). Whether JAG1 is a downstream target of Wnt signaling in ACC is currently unknown. Additionally, a mouse model of ACC in which β -catenin is constitutively active has been recently reported (10). It would be informative to examine whether β -catenin activation has an effect on Jag1 and other Notch factor expression in this model. Furthermore, conditional knockout of Jag1 in Wnt/ β -catenin-induced colorectal tumors results in a reduction in tumor size when compared to tumors in which Jag1 expression is not genetically altered (50). Understanding the mechanism of JAG1 upregulation in ACC will be an important area of investigation. Moreover, the correlation of high JAG1 levels with high grade and late stage ACC in the current study is provocative and suggests a potential novel target for therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Adrenocortical carcinoma (ACC) is a rare, highly aggressive endocrine neoplasm. Due to difficulty of detection and lack of effective treatments, ACC frequently presents with an extremely poor prognosis. Despite recent technological advancements in genetic profiling of ACC, the molecular pathogenesis of ACC has remained unclear, particularly pertaining to factors involved in late-stage disease. We report here for the first time that JAG1, a ligand for the Notch signaling pathway, is upregulated in ACC, enhances ACC cell proliferation in a non-cell-autonomous manner and is positively correlated with late-stage, aggressive ACC. JAG1 and the Notch signaling pathway may be novel targets for therapeutic intervention in late-stage ACC.

Figure 1.

JAG1 is upregulated in human ACC. A, Heatmap of Affymetrix U133A 2.0 Plus oligonucleotide array representing Notch ligand genes. Normal Adrenal (NL), n=10, Adrenocortical Adenoma (ACA) n=22, Adrenocortical Carcinoma (ACC), n=33. Scale is indicated. B, Dot plot of two JAG1 and JAG2 probe sets. Each dot indicates one tissue sample. Lines indicate mean expression levels. JAG1 #1: ACC vs NL * $p=4 \times 10^{-6}$, ACC vs ACA # $p=7 \times 10^{-12}$, JAG1 #2: ACC vs NL ** $p=1 \times 10^{-6}$, ACA vs NL ## $p=2 \times 10^{-11}$, JAG2 #1: ACC vs NL *** $p=6 \times 10^{-4}$, ACC vs ACA ### $p=4 \times 10^{-4}$, JAG2 #2: ACC vs NL **** $p=5.3 \times 10^{-3}$, ACC vs ACA ##### $p=3 \times 10^{-4}$. C, QPCR analysis of mRNA from randomly

selected human samples (NL: n=5, ACA: n=5, ACC: n=10) for JAG1 and JAG2. Each data point represents an average of triplicate determinations. *D*, Correlation of Log-transformed JAG1 QPCR expression data (from Fig 1C) with the corresponding JAG1 (probe set #2) microarray data (from Fig 1B) ($r=0.874$, $p=5\times 10^{-7}$) and JAG2 QPCR data with JAG2 (probe set #1) microarray data ($r=0.545$, $p=0.013$). *E*, Top panel: Immunoblot analysis of 5 μ g of protein lysates from randomly selected human adrenal samples (NL: n=5, ACA: n=5, ACC: n=10). Blots were probed for Jagged1 and β -actin used as a loading control. Molecular weights are indicated. Bottom panel: Quantification of Immunoblots. Jag1 protein was normalized to β -Actin and then to NL sample #06. The average of two experiments is presented. Line represent mean.

Figure 2.

The Y1 Mouse ACC Cell Line Exhibits Active Notch Signaling. **A**, Immunoblot analysis of 10 µg of protein lysate from WT mouse adrenal (Adrenal), mouse ACC cell line (Y1), and human ACC (H295A, RL251) cell lines for Jag1, Notch1, Notch2, and NICD. β-actin is used as loading control. **B**, Efficiency-corrected Δ CT QPCR method of triplicate samples used to quantify the expression of the 5 Notch ligands in Y1 cells (* JAG1 vs JAG2, $p=0.00001$). Representative experiment of three repetitions. **C**, Immunofluorescent colocalization of Jag1 with Notch1, Notch2, and Hes1 in Y1 cells. **D**, Top panel: Immunoblot analysis of 10 µg of protein lysate from Y1 cells treated with 5mM EDTA or vehicle (PBS) for 6h. Blots were probe for NICD and β-actin, used as loading control. Bottom panel: Luciferase assay of triplicate samples of Notch (4xwtCBF1Luc; pJH23A) and Control (4xmtCBF1Luc; pJH25A) reporter in Y1 cells treated with 5mM EDTA or vehicle for 6h. Luciferase expression is normalized to p-RL-TK *Renilla* expression (EDTA, Notch: * vs EDTA, Control $p=0.004$, # vs Vehicle, Notch $p=0.0039$). Representative experiment of three repetitions. **E**, Top panel: Immunoblot analysis of NICD levels of 10 µg of protein lysates of Y1 cells grown at 10%, 25%, 50%, 90% confluence. β-actin is used as loading control. Bottom panel: Luciferase assay of triplicate samples of Notch and Control reporter.

expression (normalized to *Renilla*) in Y1 cells grown at 10% (low density) and 90% (high density) confluence (High density, Notch: * vs High density, Control p=0.00003, # vs Low density, Notch p=0.0006). Representative experiment of three repetitions.

Figure 3.

Jag1 knockdown in Y1 cells inhibits proliferation in a density-dependent manner. *A*, Immunoblot analysis of protein lysates from stable cell lines expressing shRNAs for either Scramble or Jag1 [Scramble (GFP+) and Jag1KD (GFP+) respectively]. Blots were probed for Jag1 and β -actin, used as loading control. *B*, QPCR analysis of mRNA from Scramble (GFP+) and Jag1KD (GFP+) stable cell lines analyzed by the $\Delta\Delta$ CT method, and normalized to β -actin [Scramble (GFP+) vs Jag1KD (GFP+): * Jag1 $p=0.0002$, # Hes1 $p=0.001$]. Representative experiment of five repetitions. Absorbance values obtained from MTS viability assay on cell lines [Scramble (GFP+) vs Jag1KD (GFP+)] plated at (C) 10% confluence at Day 1 and growth to 35% confluence by Day 4 and (D) 40% confluence at Day 1 and growth to 85% by Day 4, Scramble (GFP+) vs Jag1KD (GFP+) at Day 4, * $p=0.0045$. Each data point represents an average $\pm$ SD of 6 determinations. Representative experiment of four repetitions.

Figure 4.

Jag1 enhances ACC proliferation in a non-cell-autonomous manner. **A**, Schematic indicating experimental design. dsRedII+ (normal Y1) cells were co-cultured with GFP+ (Scramble or Jag1KD) cells in ratios 90% Red+/10% GFP+, or 10% Red+/90% GFP+. Initial combined cell number (Red+ plus GFP+) was 150,000 cells and triplicate wells were plated. The same initial plating was used for each time point and cells were plated 4 days from harvest for the Day 4 timepoint, 3 Days from the harvest for the Day 3 timepoint, etc. Harvested cells were analyzed by FACS. 10,000 sorted cells were counted for each timepoint and the number of Red+ and GFP+ determined for each count. The percentage change in cell number from Day 1 was determined by the formulas indicated and based on the 10,000 cells counted for each

time point. *B*, Immunocytochemical images of the two different co-culture conditions at Day 2. *C*, Top panel: The percentage change of Red+ cells from Day 1 for each timepoint in the 90% Red+/10% GFP+ condition. Bottom panel: The percentage change of GFP+ cells from Day 1 for each timepoint in the 90% Red+/10% GFP+ condition. *D*, Top panel: The percentage change of Red+ cells from Day 1 for each timepoint in the 10% Red+/90% GFP+ condition (* $p < 0.03$). Bottom panel: The percentage change of GFP+ cells from Day 1 for each timepoint in the 10% Red+/90% GFP+ condition. Scramble (GFP+) vs Jag1KD (GFP+) at Day 2, 3, and 4, * $p < 0.0001$). Each bar represents an average $\pm$ SD of 3 determinations. Representative experiment of three repetitions.

Figure 5.

DNMaml suppression of Notch-dependent transcription reduces Y1 cell proliferation to a similar degree as Jag1 Knockdown. **A**, QPCR of mRNA from stable cells lines expressing either Control (GFP+) or DNMaml (GFP+) constructs analyzed using the $\Delta\Delta CT$ method and normalized to β -actin [Control (GFP+) vs DNMaml (GFP+): Hes1 * $p=0.0001$, Cdkn1a # $p=0.02$]. **B**, Absorbance values obtained from MTS viability assay on cell lines [Control (GFP+) vs DNMaml (GFP+), * $p<0.0001$]. Each data point represents an average $\pm$ SD of 6 determinations. Representative experiment of four repetitions. Co-culture of 50% normal Y1 cells (Red+) and either 50% Control (GFP+) or 50% DNMaml cells (GFP+). Initial

combined cell number (Red+ plus GFP+) was 150,000 cells and triplicate wells were plated. The same initial plating was used for each time point and cells were plated 4 days from harvest for the Day 4 timepoint, 3 Days from the harvest for the Day 3 timepoint, etc. Harvested cells were analyzed by FACS. 10,000 cells were counted for each timepoint and the number of Red+ and GFP+ determined for each count. The percentage change in cell number from Day 1 was determined by the formulas indicated (y-axis) and based on the 10,000 cells counted for each time point, *C*, Left panel: The percentage change of Red+ cells from Day 1 for each timepoint. Right panel: mRNA was harvested from Red+ cells at the Day 4 timepoint for the Control and DN Maml co-culture. Hes1 expression was determined by the $\Delta\Delta\text{CT}$ method and normalized to β -actin. *D*, Left panel: The percentage change of GFP+ cells from Day 1 for each timepoint [Control (GFP+) vs DN Maml (GFP+), * $p<0.0001$, # $p=0.06$]. Right panel: mRNA was harvested from GFP+ cells at the Day 4 timepoint for the Control and DN Maml co-culture. Hes1 expression was determined by the $\Delta\Delta\text{CT}$ method and normalized to β -actin (* $p=0.0066$). Each bar represents an average $\pm$ SD of 3 determinations. Representative experiment of three repetitions.

Figure 6.

JAG1 expression is highest in aggressive, highly proliferating ACC. *A*, Correlation of JAG1 expression (base-2 log transformed) for stage in ACCs ($n=33$). 19 Stage I + II vs 14 Stage III + IV $p=0.0551$, overall correlation $r=0.35$, $p=0.04$. *B*, Correlation of JAG1 expression (base-2 log transformed) with mitotic rate (base-2 log transformed). Overall correlation $r=0.40$, $p=0.02$. *C*, Correlation of JAG1 expression (base-2 log transformed) with KI67 expression (base-2 log transformed) across all human adrenal samples used in the microarray data set. Overall correlation $r = 0.62$, $p<0.0001$. *D*, Correlation of JAG1 expression (base-2 log transformed) with TOP2A expression (base-2 log transformed) across all human adrenal samples used in the microarray data set. Overall correlation $r=0.69$, $p<0.0001$.