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 quantitative real-time PCR (QPCR) and immunoblots of RNA and protein, respectively. ACC cells lines were assessed for Notch pathway member expression by immunoblot, QPCR, and immunofluorescence. Notch pathway activity was also determined using a reporter gene (luciferase) activation. Proliferation experiments using a Jag1 knockdown strategy (Jag1KD) and an inhibitor of Notch-dependent transcription (DNMaml) used a coculture system with fluorescence-activated cell-sorting (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 ACCs. 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 Jag1KD and coculture experiments. Inhibition of Notch signaling at the level of postreceptor signaling (DNMaml), results in similar inhibition of cell proliferation. Analysis of clinical data indicates that Jag1 expression correlates with both grade and stage of ACCs, supporting a role of JAG1-dependent Notch activation in late-stage ACCs.

Conclusions: JAG1 is the primary upregulated Notch ligand in ACCs and enhances ACC cell proliferation and tumor aggressiveness in a non-cell-autonomous manner through activation of Notch signaling in adjacent cells. Clin Cancer Res; 18(9); 2452–64. ©2012 AACR.

Introduction

Adrenocortical tumors (ACT) are extremely common neoplasms, the vast majority being benign adrenocortical adenomas (ACA) that occur in as many as 4% to 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). Because of difficulty of early detection and lack of effective treatments for advanced-stage ACCs, the average survival for surgically unresectable tumors is 12 months and the overall 5-year survival rate 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 ACCs. Specifically, the Wnt signaling pathway, a critical mediator of adrenal development, plays an important role in the etiology of ACCs, 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. Although 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 2 locations releasing the active signaling molecule NICD (cleaved Notch intracellular domain). NICD interacts with constitutively DNA-bound CSL (CBF-1/RBPjκ/Su(H)/Lag-1), recruits the essential transcriptional coactivator MAML (Mastermind-like), and...
Translational Relevance

Adrenocortical carcinoma (ACC) is a rare, highly aggressive endocrine neoplasm. Because of difficulty of detection and lack of effective treatments, ACC frequently presents with an extremely poor prognosis. Despite recent technological advancements in genetic profiling of ACCs, 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 ACCs, enhances ACC cell proliferation in a non–cell-autonomous manner, and is positively correlated with late-stage, aggressive ACCs. JAG1 and the Notch signaling pathway may be novel targets for therapeutic intervention in late-stage ACCs.

initiates transcription of Notch-dependent genes such as the HES (hairy 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 noncanonical 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 ACCs 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 carried out with Affymetrix U133A 2.0 Plus oligonucleotide arrays and have been published previously (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 on the basis of 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 209784_s_at, JAG1 Set #2 209099_x_at, JAG2

Set #1 209784_s_at, and JAG2 Set #2 32137_at. Correlations were carried out using JAG1 probe set 216268_s_at with Kil67 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 (Ann Arbor, MI) Comprehensive Cancer Center Tissue Procurement Service with Institutional Review Board approval. Samples for protein and RNA analysis were randomly selected [normal adrenal (NL): n = 5; ACA: n = 5; ACC: n = 10]. Because of tissue availability, different pools of samples were analyzed for message and protein.

Plasmids, short hairpin RNA, and transfection

Notch reporter (pH23A: 4xwtfCBF1Luc) and control reporter (pH25A: 4xmtCBF1Luc) expression vectors were a generous gift from Dr. S. Dianne Hayward (John Hopkins University Medical School, Baltimore, MD; ref. 26). The Notch reporter contains 4 consensus CSL-binding sites driving expression of firefly luciferase whereas these sites are mutated in the control reporter. pGIPZ vectors (Open Biosystems) expressing short hairpin RNA (shRNA) against JAG1 and a nonspecific scrambled control shRNA (scramble) were obtained from the University of Michigan shRNA core (http://www.med.umich.edu/vcore/vector-shRNA.htm). In addition to the shRNA, pGIPZ vectors contain a puromycin selection cassette and an IRES GFP sequence. Sequences for JAG1 shRNA are #1: 5'-gtcaaatggccataaa-3' and #2: 5'-ggatttggttaatggtta-3'. pdsREDII expresses dsREDII under control of the cytomegalovirus (CMV) promoter and was obtained from Dr. Claudius Vincenz (University of Michigan). Control (MigR1) plasmid, which expresses GFP, and DNAMaml plasmid, which expresses a fusion protein of GFP and amino acids 13 to 74 of Maml1 and acts as a dominant-negative, were a generous gift of Dr. Ivan Maillard (University of Michigan; refs. 27, 28). Both control (MigR1) and DNAMaml plasmids contain flanking long terminal repeat (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, United Kingdom).

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. Virus-competent 293T cells, a gift from Dr. Benjamin Margolis (University of Michigan), were maintained in Dulbecco’s Modified Eagle’s Media (DMEM) with 10% cosmic calf serum (CCS; Hyclone) and penicillin/streptomycin. In some experiments, Y1 cells were treated for 6 hours with 5 mmol/L EDTA prepared in PBS. Transient
transfections were carried out using FuGENE (Roche) according to the manufacturer's instructions and optimized at a 4:1 ratio (4 μL FuGENE/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 earlier, followed by 4 weeks of puromycin selection (2 μg/mL; Roche). Cells were then enriched for GFP expression within the 10⁴ to 10⁵ range using fluorescence-activated cell sorting (FACS) as described later. Sorted cells were replated in 10-cm dishes and allowed to expand.

To obtain the dsREDII (wildtype Y1 (Red⁺)) cell line, 10-cm dishes of Y1 cells were transiently transfected with 2 μg of pdsREDII as described. Because pdsREDII lacks a mammalian selection cassette, cells were passed after 2 days and were transiently transfected an additional time. After 2 days, cells were enriched for dsREDII expression within the 10⁴ to 10⁵ range using FACS. Sorted cells were replated in 10-cm 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 µg of pGIPZ vectors expressing shRNA directed against Jag1 or a control (mRNA) or DNMaml constructs. After 2 days, medium was collected and centrifuged at 5,000 × g followed by filtration through a 0.22-μm syringe nylon filter (Fisher Scientific). Viral supernatant was adjusted to a final volume of 10 mL with DMEM and polybrene was added (10 U/mL; Sigma). 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 10⁴ to 10⁵ range using FACS and were replated into 10-cm dishes and allowed to expand. Because the GFP and dsREDII expression evidently diminished overtime, cells were resorted under identical parameters every 3 months.

FACS and analysis
Trypsinized cells were pelleted at 1,000 × g for 5 minutes and resuspended in 1 × PBS containing 10% CCS at a concentration of 1 to 2 million cells/mL. FACS experiments were done 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: 488, 350, and 633 nm) or BD Biosciences FACSAria High-Speed Cell Sorter (3-laser: 488, 407, and 633 nm).

Quantitative real-time PCR analysis
RNA was isolated with TRizol (Invitrogen) according the manufacturer's instructions, and cDNA was generated using iScript cDNA Synthesis kit (Bio-Rad Laboratories). Quantitative real-time PCR (QPCR) experiments were carried out as previously described (33, 34). A comprehensive list of human and mouse QPCR primers is found in Supplementary Table S1. Analysis was conducted with either the efficiency-corrected ΔCT method or the ΔΔCT 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 and immunoblots, see Supplementary Table S2. For immunocytochemical localization: Y1 cells were plated on glass slides coated with fibronectin (10 μg/mL; Sigma). Slides were washed in 1 × PBS, fixed in 4% paraformaldehyde (Fisher) for 15 minutes at 4°C, and permeabilized with 0.02% Igepal CA-630 (Sigma). Slides were blocked with 2% milk in 1 × PBS, and primary/secondary antibodies (Supplementary Table S2) were diluted in 0.2% milk in 1 × PBS. For detection of native fluorescence, slides were not fixed to preserve the activity of GFP and dsRedII. Coverslips were applied and images obtained as previously described (33, 34).

Immunoblot analysis of protein lysates from cell cultures were conducted 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), secondary antibodies used were Odyssey IRdyes (Supplementary Table S2). Immunoblots using protein lysate from human adrenal tumor samples were quantified using ImageJ software (NIH, 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 Department of Biological Sciences (University of Iowa, Iowa City, IA).

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

 Coculture experiments
Stable cell lines of Y1 cells expressing dsREDII (wildtype Y1 (Red⁺)) were cocultured with either scramble (GFP⁺) or Jag1KD (GFP⁺) cells in triplicate wells of a 6-well plate in 2 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 per 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, and so on. At the end of the 4-day time course, harvested cells were analyzed by FACS as described earlier. Ten thousand 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, see Fig. 4A). Identical conditions and analysis were carried out for coculture of wild-type Y1 (Red⁺)
with either control (GFP+) or DNAML (GFP+) except that a 50%/50% ratio was used.

Luciferase assays
Y1 cells were plated in 24-well plates and were transiently transfected with 50 ng of pRL-TK Renilla Luciferase (Promega Corp.) and 0.5 μg of either control reporter (pH25A) or Notch reporter (pH23A) firefly luciferase constructs described earlier. Assays were conducted 24 hours after transfection using the Dual-Luciferase Reporter Assay (Promega) according to the manufacturer’s 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 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 ACCs, our group has previously conducted DNA microarray analyses using frozen human tissues—most recently with a total of 33 ACC, 22 ACA, and 10 normal adrenals (24, 36). Analysis of differentially expressed probe sets revealed an upregulation of the Notch ligand JAG1 in ACC samples compared with normal and adenomatous tissue (Fig. 1A). The 5 JAG1 probes sets depicted are within the top 0.8% of all rank-ordered upregulated probe sets represented in the microarray. The other 4 Notch ligands (JAG2, DLL1/3/4) were upregulated in only a few ACCs.

Quantitative analysis of 2 independent probe sets for each JAG ligand (JAG1 and JAG2) confirmed that JAG1 expression is significantly higher in ACC samples than in normal adrenals and ACA (Fig. 1B, top). JAG2 exhibits a statistically significant, albeit less dramatic, difference in expression among samples (Fig. 1B, bottom). Furthermore, interrogating 2 other adenocal tumor microarray data sets revealed a similar upregulation of JAG1 in ACCs (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 sample is shown in Fig. 1D. Although both JAG1 and JAG2 were significantly different in ACC versus ACA/normal adrenals, JAG1 is expressed at a higher level and a greater increase in ACC than JAG2. In addition, JAG1 QPCR expression was more tightly correlated to the microarray data (JAG1: r = 0.874, JAG2: r = 0.545). These data support the validity and biologic 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 than in normal adrenals and ACA (Fig. 1E, top). Quantification of band intensity of 2 immunoblots using the same set of human samples identifies robust protein levels of JAG1 in most ACCs and barely detectable quantities in ACAs and normal adrenals (Fig. 1E, bottom). Together, these data suggest that JAG1 mRNA and protein is upregulated in a majority of ACC samples and is consistent with biologic relevance of JAG1-activated Notch signaling contributing to adrenocortical carcinogenesis. Although 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 2 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 shows that the Y1 cell recapitulates the Notch ligand expression profile observed in the human ACC microarray where the expression of the other 4 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 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 (i.e., 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 ACCs. Because Mg2+ is required for Notch receptor stability, Mg2+ depletion can be used to induce Notch receptor cleavage and biochemically release the active NICD peptide 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 Mg2+ resulted in an increase in NICD protein in EDTA-treated cells compared with vehicle-treated cells (Fig. 2D, top). H295A human ACC cells also exhibited a similar response to EDTA treatment (Supplementary Fig. S1). To confirm that NICD cleavage results in productive Notch-dependent transduction, Y1 cells were transiently transfected with a specific Notch luciferase reporter containing 4 NICD consensus-binding sites or an otherwise identical reporter in which the NICD sites are mutated. After EDTA treatment, an
increase in the expression of the Notch luciferase reporter was observed (EDTA-Notch vs. vehicle-Notch = 2.3-fold increase; Fig. 2D, bottom). These data indicate that canonical Notch signaling can be activated in Y1 cells, presumably because of the presence of Jag1.

Because Notch signaling is generally dependent upon the juxtaposition of 2 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%, and 90% confluence), a density-dependent increase in NICD protein was observed (Fig. 2E, top). When the transcriptional activity of the Notch reporter was evaluated by luciferase assay, an elevated activity was observed in the highest (90%) when compared with the lowest (10%) density (high-density Notch versus low-density Notch = 2.3-fold; Fig. 2E, bottom). In addition, 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 that active Notch signaling is occurring in Y1 cells.

To investigate the dependence of Notch activation on Jag1 in ACC cells, an shRNA knockdown strategy was used. 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 nonspecific 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 3 weeks as determined by immunoblot analysis (Fig. 3A). Jag1 mRNA expression was also reduced by 63%, although 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 used. Although 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 with scramble (GFP⁺).
whereas cleaved caspase-3 (Clv-Csp-3), a marker of apoptosis, was not detected in either cell line (Supplementary Fig. S2A and data not shown). These data confirm that a loss of Jag1 protein inhibits the proliferation of Y1 cells. Predicted 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 biologic functions of Jag1 and the related Notch ligands DLL1 and DLL3 within the cells in which they are expressed (cell-autonomous; refs. 23, 40, 41), a coculture system was designed using 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 wild-type Y1 cells expressing dsRedII [wild-type Y1 (Red+ ); Fig. 4A]. Wild-type Y1 (Red+ ) cells were cultured with scramble (GFP+ ) or Jag1KD (GFP+ ) in 2 different ratios: 90% Red+/10% GFP+ or 10% Red+/90% GFP+ (Fig. 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 wild-type Y1 (Red+ ), whereas the latter condition (10% Red+/90% GFP+) interrogates the effect of decreased Jag1 input to wild-type Y1 (Red+) cells. To guarantee sufficient cellular interactions, cells were initially plated at high density (70% confluence by day 2; Fig. 4B). To assure that all cells could be analyzed at the same time at the end of the 4-day time course, the same initial concentration of cells was plated 4 days before analysis for day 4, 3 days before analysis for day 3, and so on (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 conducted. In the 90% Red+/10% GFP+ ratio, Jag1KD (GFP+) cells were cultured with an abundance of wild-type 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 wild-type Y1 (Red+) cells (Fig. 4B, left). Under these conditions, wild-type Y1 (Red+) cells show no relative change in proliferation whether cultured with scramble (GFP+) and Jag1KD (GFP+) cells. This is expected because wild-type Y1 (Red+) cells are most likely receiving Jag1 inputs predominantly from other wild-type Y1 (Red+) cells (Fig. 4C, top; Supplementary Fig. S3). Importantly, no difference in the proliferation of scramble (GFP+) and Jag1KD (GFP+) cells are observed under these coculture conditions (Fig. 4C, bottom; Supplementary Fig. S3). These data indicate that the scramble (GFP+) and Jag1KD (GFP+) cells are capable of receiving Jag1 inputs from wild-type Y1 (Red+) cells and, hence, proliferate, normally supporting the hypothesis that Jag1 has a non–cell-autonomous effect (Supplementary Fig. S3).

To further test the hypothesis that Jag1 acts in a non–cell-autonomous manner on adjacent cells, wild-type Y1...
(Red⁺) cells were cultured with Jag1KD (GFP⁺) or scramble (GFP⁺) cells at the ratio of 10% Red⁺/90% GFP⁻ (Fig. 4B, right). Under these conditions, wild-type Y1 (Red⁺) cells receive a majority of signaling input from GFP⁺ (scramble or Jag1KD) cells. Specifically, the wild-type Y1 (Red⁺) cells receive numerous Jag1 inputs from scramble (GFP⁺) cells and reduced Jag1 inputs from the Jag1KD (GFP⁺) cells (right, Fig. 4B; Supplementary Fig. S3). Under these conditions, wild-type Y1 (Red⁺) cells show a 23% reduction in proliferation at day 2 and a 27% reduction in proliferation at day 3 [when cocultured with Jag1KD (GFP⁺) cells (Fig. 4D, top)]. The Jag1KD (GFP⁺) cells also exhibit a maximal 35% reduction at day 4 (Fig. 4D, bottom) predicated on the assumption that they are receiving the majority of signaling inputs from neighboring Jag1KD (GFP⁺) cells.

In summary, wild-type Y1 (Red⁺) cells proliferate less well when cocultured with 90% Jag1KD (GFP⁺) cells, suggesting that a decrease in Jag1 inputs results in retarded Y1 cell growth. Jag1KD (GFP⁺) cells remain competent to receive Jag1 inputs from wild-type Y1 (Red⁺) cells as reflected in the increased proliferation of the Jag1KD (GFP⁺) cells grown in the presence of 90% wild-type Y1 (Red⁺) cells. Jag1KD (GFP⁺) cells proliferated less well in the coculture containing 90% Jag1KD (GFP⁺) cells, suggesting that the decrease in Jag1 inputs results in retarded Jag1KD (GFP⁺) cell growth. Together, the coculture studies indicate that Jag1 enhances ACC cell...
proliferation in a non–cell-autonomous manner. See experimental model in Supplementary Fig. S3.

**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 knockout 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 MAML1–4 (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 used [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 *Cdh11a* are reduced by 64% and 43%, respectively (Fig. 5A). Two unrelated but highly expressed genes in Y1 cells, *Cnmbl* (β-catenin) and *Sf1* (steroidogenic factor 1), were unaffected, consistent with a specific inhibition of Notch target genes in the DNMaml (GFP⁺) cell line.

Because DNMaml inhibits Notch-dependent transcription, we hypothesized that DNMaml (GFP⁺) cells would have a reduced ability to proliferate when compared with control (GFP⁺) cells. Using the MTS viability assay to assess proliferation, DNMaml (GFP⁺) cells plated at 40% confluency showed a 37% reduction in proliferation when compared with control (GFP⁺) cells (Fig. 5B). Furthermore, immunoblot analysis revealed a reduction in PCNA protein level of cleaved caspase-6 (Clv-Csp-6), another marker of apoptosis, was unchanged (Supplementary Fig. S2B).

Because DNMaml inhibits Notch-dependent transcription, we hypothesized that DNMaml (GFP⁺) cells would have a reduced ability to proliferate when compared with control (GFP⁺) cells. Using the MTS viability assay to assess proliferation, DNMaml (GFP⁺) cells plated at 40% confluency showed a 37% reduction in proliferation when compared with control (GFP⁺) cells (Fig. 5B). Furthermore, immunoblot analysis revealed a reduction in PCNA protein level of cleaved caspase-6 (Clv-Csp-6), another marker of apoptosis, was unchanged (Supplementary Fig. S2B).

Although 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 coculture study was carried out using a 50% wild-type Y1 (Red⁺)/50% control (GFP⁺) or DNMaml (GFP⁺) ratio. Wild-type Y1 (Red⁺) cells cocultured with either control (GFP⁺) or DNMaml (GFP⁺) cells maintain robust proliferation (Fig. 5C, left). No statistically significant difference in *Hes1* expression was observed on day 4 in wild-type Y1 (Red⁺) cells cultured with GFP⁺ (control or DNMaml) cells, indicating that DNMaml is not affecting Notch signaling in adjacent wild-type Y1 (Red⁺) cells (Fig. 5C, right). Conversely, DNMaml (GFP⁺) cells cultured with wild-type Y1 (Red⁺) cells exhibit a 34.3% reduction in proliferation when compared with control (GFP⁺) cells cultured with wild-type Y1 (Red⁺) cells (left, Fig. 5D). *Hes1* mRNA was reduced 71.34% in DNMaml (GFP⁺) versus control (GFP⁺) cells at day 4 (Fig. 5D, right). These data indicate DNMaml is acting specifically in DNMaml (GFP⁺) cells. Together with the Jag1 coculture 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 ACCs and acts through canonical Notch signaling to enhance density-dependent ACC cell proliferation. To determine whether elevated JAG1 mRNA expression levels in human ACCs 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 and 6B). Specifically, JAG1 expression was increased 1.67-fold (P = 0.05) in late-stage ACCs (stages III and IV) compared with early-stage ACCs (stages I and II; Fig. 6A). Our previous microarray has shown strong correlations between *Ki67* and topoisomerase 2A (TOP2A) expression, 2 markers of proliferation that are highly upregulated in ACCs, 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 ACCs. 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 coculture experiments. Inhibition of Notch signaling at the level of ligand (Jag1KD) or postreceptor signaling (DNMaml) results in similar inhibition of cell proliferation. Analysis of clinical data indicates that Jag1 expression correlates with both grade and stage of ACCs, supporting a role of JAG1-dependent Notch activation in ACCs.

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 cyclin D1 in prostate cancer (21), increase the number of cancer cells in S-phase (41), and facilitate proliferation in Wnt1-transformed breast epithelial cells (42, 43).

Although the canonical mechanism by which Jag1 mediates cellular effects in numerous systems is through
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 \( \Delta \Delta C_T \) method and normalized to \( \beta \)-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 4 repetitions. Coculture 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 time point, 3 days from the harvest for the day 3 time point, and so on. Harvested cells were analyzed by FACS. Ten thousand cells were counted for each time point and the number of Red+ and GFP+ were 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, the percentage change of Red+ cells from day 1 for each time point; right, mRNA was harvested from Red+ cells at the day 4 time point for the control and DNMaml coculture. Hes1 expression was determined by the \( \Delta \Delta C_T \) method and normalized to \( \beta \)-actin \( \). D, left, the percentage change of GFP+ cells from day 1 for each time point [control (GFP+) vs. DNMaml (GFP+)] \( P < 0.0001; \) #, \( P = 0.06 \); right, mRNA was harvested from GFP+ cells at the day 4 time point for the control and DNMaml coculture. Hes1 expression was determined by the \( \Delta \Delta C_T \) method and normalized to \( \beta \)-actin \( P = 0.0066 \). Each bar represents an average \( \pm \) SD of 3 determinations. Representative experiment of 3 repetitions.
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 using specific shRNAs resulted in a density-dependent reduction in proliferation. Coculture experiments of normal Y1 cells with either Jag1KD or scrambled 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 that 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 after inhibition of Notch-dependent transcription using 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 ACCs. 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/b-catenin signaling on the formation of the otic placode (46). Moreover, Jag1 has been shown to be a direct target of b-catenin in the epidermis where Notch signaling is required for b-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 ACCs is unknown. Although repression of Notch-dependent transcription had no effect on b-catenin (Ctnnb1) expression in the DNMaml experiments (Fig. 5A), it remains unknown whether Wnt activation synergizes or activates various components of the Notch pathway. Nuclear b-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 ACCs is currently unknown. In addition, a mouse model of ACC in which b-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 with 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 this study is provocative and suggests a potential novel target for therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.P. Simon, T.J. Giordano, G.D. Hammer

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Upregulated JAG1 Enhances Cell Proliferation in Adrenocortical Carcinoma

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