Targeting the Notch Ligand Jagged1 in Both Tumor Cells and Stroma in Ovarian Cancer

Adam D. Steg¹, Ashwini A. Katre¹, Blake Goodman², Hee-Dong Han²,⁵, Alpa M. Nick², Rebecca L. Stone², Robert L. Coleman², Ronald D. Alvarez¹, Gabriel Lopez-Berestein³,⁵, Anil K. Sood²,⁴,⁵ and Charles N. Landen¹*

¹Department of Obstetrics and Gynecology, University of Alabama at Birmingham, Birmingham, AL 35294
²Department of Gynecologic Oncology, U.T.M.D. Anderson Cancer Center, 1155 Herman Pressler Boulevard, Unit 1362, Houston, TX 77030
³Department of Experimental Therapeutics, U.T.M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 422, Houston, TX 77030
⁴Department of Cancer Biology, U.T.M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 173, Houston, TX 77030
⁵Center for RNA Interference and Non-Coding RNA, U.T.M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030

Running Title: Targeting Jagged1 in ovarian cancer
Keywords: Jagged1, chemoresistance, small interfering RNA, ovarian cancer
Acknowledgements: Funding support provided by the Reproductive Scientist Development Program through the Ovarian Cancer Research Fund and the National Institutes of Health (K12 HD00849), the Gynecologic Cancer Foundation and the Department of Defense Ovarian Cancer Research Academy (OC093443) to CNL; and NIH (CA10929801, P50 CA083639, CA128797, RC2GM092599, U54 CA151668), and the Ovarian Cancer Research Fund, Inc. (Program Project Development Grant) grants to AKS.

*Correspondence: Charles N. Landen, Jr., MD, MS
Assistant Professor
Department of Obstetrics and Gynecology
The University of Alabama at Birmingham
1825 University Boulevard
505 Shelby Building
Birmingham, AL 35294
Work: 205-934-0473
Fax: 205-934-0474
clanden@uab.edu
TRANSLATIONAL RELEVANCE

Most ovarian cancer patients will have an excellent response to initial surgical debulking and chemotherapy, but about 75% of patients will later recur and succumb to disease. There is likely a privileged population with either inherent or acquired resistance to chemotherapy, and finding therapies against this population is essential to achieving long-term disease control. Among many potential pathways implicated in survival of these populations is the Notch pathway, and the Notch ligand Jagged1, which can be expressed on both tumor endothelial cells and tumor cells. We have demonstrated that Jagged1 plays important roles in both compartments of the tumor – chemoresistance in tumor cells, and angiogenesis in stroma. Furthermore, targeting Jagged1 independently on endothelial cells leads to reduced angiogenesis and, on tumor cells, reduces proliferation, and reverses taxane resistance. A novel mechanism of Jagged1 signaling through the Hedgehog pathway is also described. Our findings highlight the importance that dual targeting of each compartment of the tumor microenvironment (stromal and malignant cells) is an important principle underlying therapy, and that therapies specifically against Jagged1 may improve response rates and outcomes in ovarian cancer.
ABSTRACT

Purpose: Jagged1, a Notch ligand, is expressed on both tumor epithelial and endothelial cells, and therefore may be amenable to dual targeting of the tumor stroma and malignant cell compartments of the tumor microenvironment. Experimental Design: We describe in vitro effects of targeting of Jagged1 on ovarian cancer cells and in vivo effects of independent targeting of stromal and malignant cell Jagged1 using species-specific human or murine siRNA constructs incorporated into chitosan nanoparticles (CH) and delivered intravenously in an orthotopic mouse model. Results: Jagged1 expression was prominent in SKOV3ip1, and IGROV-AF1, and significantly overexpressed in SKOV3TRip2, a taxane-resistant SKOV3 subclone. Jagged1 silencing with siRNA decreased cell viability and reversed taxane chemoresistance. In two different orthotopic ovarian cancer models, treatment with anti-human Jagged1 siRNA-CH reduced growth by 54.4-58.3%, and with anti-murine Jagged1 siRNA-CH reduced growth by 41.7-48.8%. The combination of both species-specific constructs reduced tumor weight by 87.5-93.1% and sensitized SKOV3TRip2 tumors to docetaxel in vivo. Tumors demonstrated reduced microvessel density with anti-murine Jagged1 constructs, and decreased proliferation with anti-human Jagged1 siRNAs-CH. In addition, we show that Jagged1 downregulation does not sensitize cells to taxanes through a reduction in MDR1 expression, but at least in part by crosstalk with the GLI2 mediator of the Hedgehog pathway. Conclusions: Jagged1 plays dual roles in cancer progression, through an angiogenic function in tumor endothelial cells, and through proliferation and chemoresistance in tumor cells. Dual inhibition represents an attractive therapeutic strategy for ovarian and potentially other malignancies.
INTRODUCTION

Ovarian cancer is the leading cause of death from a gynecologic malignancy. Although ovarian cancer is among the most chemosensitive malignancies at the time of initial treatment (surgery and taxane/platinum-based chemotherapy), most patients will develop tumor recurrence and succumb to chemoresistant disease (1). Evaluation of multiple chemotherapy agents in several combinations in the last 20 years has yielded modest improvements in progression-free survival, but no increases in durable cures. The clinical course suggests that a population of tumor cells has either inherent or acquired resistance to chemotherapy that allows survival with initial therapy and ultimately leads to recurrence. Targeting the cellular pathways involved in this resistance may provide new treatment modalities for ovarian cancer.

The Notch pathway plays an important role in cell growth and differentiation during embryonic development (2). Mature Notch receptors (Notch1, 2, 3 and 4) consist of an extracellular and transmembrane unit. Upon binding to ligands (Jagged1, 2 and Delta-like ligand (DLL) 1, 3 and 4) on the surface of neighboring cells, the Notch extracellular unit is dissociated from the transmembrane unit, which is then endocytosed into ligand-expressing cells. Further cleavage of the transmembrane unit by “a disintegrin and metalloprotease” proteins (ADAM10 and/or 17) and γ-secretase produces an active, intracellular fragment that translocates to the nucleus, where it forms a transcriptional complex with CSL, Mastermind-like proteins (MAML1, 2 and 3) and p300. Transcriptional mediators of Notch signaling include members of the HES and HEY family. Recent reports have implicated Notch signaling in multiple malignancies (3), including ovarian cancer (4-7), and suggest this pathway may be especially important.
in maintaining the subpopulation of cancer cells with stem cell properties (8) as well as conferring resistance to chemotherapies (9-10). The Notch ligand Jagged1 is frequently overexpressed on both ovarian cancer cells (6) and tumor-associated endothelial cells (11), suggesting that selectively targeting this protein may present a novel therapeutic strategy to target both stromal and tumor cells in ovarian cancer. The Notch pathway is highly implicated in normal and tumor-associated angiogenesis (12-14). Moreover, studies have shown that Jagged1 can activate gene expression (15) and transform kidney epithelial cells (16) without involvement of Notch signaling, indicating that Jagged1 may have its own signaling function that is important to tumorigenesis independent of the canonical Notch pathway.

As most studies have focused on the effects of inhibiting the Notch receptor and its downstream signaling, specific inhibition of Jagged1 has not been fully explored, which is especially important given potential Notch-independent effects of Jagged1. Additionally, it is not known whether the greater *in vivo* contribution of Notch inhibition is through its anti-angiogenic mechanism or specific activity against malignant cells. In the current study, we sought to determine the effects of targeting Jagged1 on the viability and taxane and platinum chemoresistance of ovarian cancer cells and tumor-associated stroma, independently and concurrently. Utilizing a novel methodology for delivering siRNA *in vivo*, we independently target Jagged1 in stromal cells with anti-murine siRNA and Jagged1 in malignant cells with anti-human siRNA, and demonstrate that Jagged1 plays important roles in tumor angiogenesis and chemoresistance and is an attractive target for therapy.
METHODS

Cell lines and culture. The ovarian cancer cell lines A2780ip2, A2780cp20, HeyA8, HeyA8MDR, IGROV-AF1, SKOV3ip1 and SKOV3TRip2 (17-18) were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). A2780cp20 (platinum-resistant), HeyA8MDR (taxane-resistant) and SKOV3TRip2 (taxane-resistant, a kind gift of Dr. Michael Seiden (19)) were generated by sequential exposure to increasing concentrations of chemotherapy. HeyA8MDR and SKTRip2 were maintained with the addition of 150 ng/ml of paclitaxel. The murine ovarian endothelial cell (MOEC) line was established from the immortomouse, which harbors temperature-sensitive SV40 large T antigen overexpression (20). MOEC cells allowed for the evaluation of murine-specific Jagged1 expression. All cell lines were routinely screened for Mycoplasma species (GenProbe detection kit; Fisher, Itasca, IL) with experiments performed at 70-80% confluent cultures. Purity of cell lines was confirmed with STR genomic analysis, and only cells less than 20 passages from stocks were used in experiments.

RNA extraction and reverse transcription. Total RNA was isolated from ovarian cancer cell lines using Trizol reagent (Invitrogen, Carlsbad, CA) per manufacturer’s instructions. RNA was then DNase treated and purified using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA was eluted in 50 μL of RNase-free water and stored at -80°C. The concentration of all RNA samples was quantified by spectrophotometric absorbance at 260/280 nm using an Eppendorf BioPhotometer plus (Hamburg, Germany). Prior to cDNA synthesis, all RNA samples were diluted to 20 ng/μL using RNase-free
water. cDNA was prepared using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The resulting cDNA samples were analyzed using quantitative PCR.

**Quantitative PCR.** Primer and probe sets for *GLI1* (Hs00171790_m1), *GLI2* (Hs00257977_m1), *HES1* (Hs00172878_m1), *HEY1* (Hs00232618_m1), *JAG1* (Hs01070032_m1), *MDR1* (Hs00184500_m1), *NOTCH1* (Hs00413187_m1), *NOTCH3* (Hs01128541_m1) and *RPLP0* (Hs99999902_m1; housekeeping gene) were obtained from Applied Biosystems and used according to manufacturer’s instructions. PCR amplification was performed on an ABI Prism 7900HT sequence detection system and gene expression was calculated using the comparative $C_T$ method as previously described (21). Briefly, this technique uses the formula $2^{-\Delta\Delta C_T}$ to calculate the expression of target genes normalized to a calibrator. The cycling threshold ($C_T$) indicates the cycle number at which the amount of amplified target reaches a fixed threshold. $C_T$ values range from 0 to 40 (the latter representing the default upper limit PCR cycle number that defines failure to detect a signal).

**Western blot analysis.** Cultured cell lysates were collected in modified radioimmunoprecipitation assay (RIPA) lysis buffer with protease inhibitor cocktail (Roche, Manheim, Germany) and subjected to immunoblot analysis by standard techniques (22) using anti-Jagged1 antibody (28H8, Cell Signaling Technology, Danvers, MA) at 1:1000 dilution overnight at 4°C; anti-Poly (ADP-ribose) polymerase (PARP) antibody (7D3-6, BD Biosciences, San Jose, CA) at 1:1000 dilution overnight at 4°C;
anti-Cleaved Notch1 antibody (Cell Signaling Technology, Danvers, MA) at 1:1000 dilution overnight at 4°C; anti-Notch3 antibody (M-134, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 dilution overnight at 4°C; anti-Gli2 antibody (Sigma, St. Louis, MO) at 1:500 dilution overnight at 4°C; or anti-β-actin antibody (AC-15, Sigma) at 1:20,000 dilution for 1 hour at RT, which was used to monitor equal sample loading. After washing, blots were incubated with goat anti-rabbit (for Jagged1, Notch1, Notch3 and Gli2) or goat anti-mouse (for PARP and β-actin) secondary antibodies (Bio-Rad, Hercules, CA) conjugated with horseradish peroxidase. Visualization was performed by the enhanced chemiluminescence method (Pierce Thermo Scientific, Rockford, IL).

**siRNA transfection.** To examine downregulation of Jagged1, Gli2, Notch1 or Notch3 with siRNA, cells were exposed to control siRNA (target sequence: 5'-UUCUCCGAACGUGUCACGU-3', Sigma), or one of 2 tested Jagged1-targeting constructs (JAG1_A: 5'-GAAUGUGAGGCCAAACCUCU-3' or JAG1_B: 5'-CCUGUAACAUAGCCCGAAA-3', Sigma), or one of 2 tested Gli2-targeting constructs (GLI2_A: 5'-GUACCAUUACGAGCCUCAU-3' or GLI2_B: 5'-CCUUCAAGGCGCAGUACAU-3', Sigma), or a Notch1-targeting construct (5'-GUGUGAAUCCACCCUUGU-3', Sigma), or a Notch3-targeting construct(5'-GGUAGUAAUGCUGAGAUU-3', Sigma) at a 1:3 siRNA (µg) to Lipofectamine 2000 (µL) ratio. Lipofectamine and siRNA were incubated for 20 min at RT, added to cells in serum-free RPMI to incubate for 6 hours, followed by 10% FBS/RMPl thereafter. Transfected cells were grown at 37°C for 48-72 hours and then harvested for viable cell counting, quantitative PCR or Western blot analysis.
Assessment of cell viability with chemotherapy IC50 and cell cycle analysis. To a 96-well plate, 2,000 cells/well were exposed to increasing concentrations of docetaxel in triplicate. Viability was assessed with 0.15% MTT (Sigma). For effects of siRNA-mediated downregulation on docetaxel IC50, cells were first transfected with siRNA (5 µg) for 24 hours in 6-well plates, then trypsinized and re-plated at 2,000 cells per well, followed by addition of chemotherapy after attachment. IC50 was determined by finding the dose at which the drug had 50% of its effect, calculated by the equation \[ (\text{OD}_{450_{\text{MAX}}}-\text{OD}_{450_{\text{MIN}}})/2 + \text{OD}_{450_{\text{MIN}}} \]. Test of synergy was performed by the Loewe additivity model (23), calculated by the equation \[ CI = [D_1/D_{x1}] + [D_2/D_{x2}] \], where a CI (combination index) of 1 suggests an additive effect, <1 suggests synergy, and >1 suggests antagonism. For cell cycle analysis, cells were transfected with siRNA for 72 hours, trypsinized, and fixed in 75% ethanol overnight. Cells were then centrifuged, washed x2 in PBS, and reconstituted in PBS with 50 µg/mL propidium iodide (PI). PI fluorescence was assessed by flow cytometry and the percentage of cells in sub-G0, G0/G1, S and G2/M phases were calculated by the cell cycle analysis module for Flow Cytometry Analysis Software (FlowJo v.7.6.1, Ashland, OR).

Orthotopic ovarian cancer model and in vivo delivery of siRNA. For orthotopic therapy experiments using ovarian cancer cell lines, female athymic nude mice (NCr-nu) were purchased from the National Cancer Institute (Frederick, MD) after Institution Animal Care and Use Committee approval of protocols, and cared for in accordance with guidelines of the American Association for Accreditation of Laboratory Animal Care. For all in vivo experiments, trypsinized cells were resuspended in 10% FBS-containing
RPMI, washed with PBS, and suspended in serum-free HBSS at a concentration of 5 x 10^6 cells/mL, and 1 x 10^6 cells (IGROV-AF1 or SKOV3TRip2) were injected IP in 200 μL into 40 mice per experiment. After 1 week, mice (n=10 per group) were randomized to treatment with a) 10 μg control siRNA; b) 5 μg murine-specific anti-Jagged1 siRNA (target sequence: 5’-CAGUAAUGACACUAUUCAA-3’, Sigma) plus 5 μg control siRNA; c) 5 μg human-specific anti-Jagged1 siRNA (same as JAG1_B siRNA) plus 5 μg control siRNA; or d) 5 μg of both species-specific siRNA constructs. In another experiment, mice bearing SKOV3TRip2 tumors were randomized to treatment with a) 10 μg control siRNA; b) 10 μg control siRNA plus docetaxel; c) both species-specific anti-Jagged1 siRNA constructs (5 μg each); or d) both species-specific constructs (5 μg each) plus docetaxel. In a separate experiment, mice bearing SKOV3TRip2 tumors were randomized to treatment with a) 10 μg control siRNA plus docetaxel; b) 5 μg human-specific anti-Jagged1 siRNA plus 5 μg control siRNA plus docetaxel; c) 5 μg murine-specific anti-Jagged1 siRNA plus 5 μg control siRNA plus docetaxel; or d) both species-specific constructs (5 μg each) plus docetaxel. siRNA constructs were incorporated in chitosan nanoparticles (CH) as previously described (24-25) and administered IV twice per week in a volume of 100 μL. Docetaxel was administered IP at a dose of 35 μg weekly. Mice were treated for 4-6 weeks before sacrifice and tumor collection.

**Immunohistochemical staining.** Resected tumors frozen in Tissue-Tek OCT compound (Sakura, Torrance, CA) were used for immunohistochemical (IHC) analysis of microvessel density (MVD) using anti-CD31 antibodies (Cell Signaling Technology). Analysis of cell proliferation was determined by IHC performed on formalin-fixed
paraffin-embedded (FFPE) tumors with anti-PCNA antibodies (Cell Signaling Technology). Detection of CD31 and PCNA were carried out as previously described (26). To quantify MVD and cell proliferation, five random fields were recorded for each tumor at x100 magnification. A vessel was defined as CD31 staining with a visible associated lumen. PCNA staining was considered positive if the entire nucleus was strongly positive. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed as previously described to determine cell apoptosis (27). Quantification of apoptosis was calculated by determining the number of apoptotic cells in five random fields for each tumor at x200 magnification. All staining was quantified by two investigators in a blinded fashion. Images were assessed and quantified without modification, however for publication of PCNA figures, contrast was enhanced to an entire image using the “Auto Contrast” tool in Photoshop, to avoid bias.

**Statistical analysis.** Comparisons of gene expression, PI fluorescence, mean tumor weight and mean MVD, PCNA and TUNEL+ cells were analyzed using a two-tailed Student’s t-test, if assumptions of data normality were met. Those represented by alternate distribution were examined using a nonparametric Mann-Whitney U test. Differences between groups were considered statistically significant at p<0.05. Error bars represent standard deviation unless otherwise stated. Number of mice per group (n=10) was chosen as directed by a power analysis to detect a 50% decrease in tumor growth with beta error of 0.2.
RESULTS

**Jagged1 expression in ovarian cancer cell lines.** We first examined Jagged1 expression (both mRNA and protein) in IGROV-AF1 and 3 pairs of parental and chemoresistant ovarian cancer cell lines: A2780ip2 / A2780cp20 (20-fold increased cisplatin resistance and 10-fold increased taxane resistance); HeyA8 / HeyA8MDR (500-fold taxane resistant); and SKOV3ip1 / SKOV3TRip2 (1000-fold taxane resistant). mRNA expression of Jagged1, as measured by quantitative PCR, was prominent in IGROV-AF1, SKOV3ip1 and SKOV3TRip2 with little to no expression in the A2780ip2 / A2780cp20 and HeyA8 / HeyA8MDR lines (Figure 1A). Western blot analysis demonstrated similar Jagged1 expression as that obtained using qPCR (Figure 1B). Interestingly, Jagged1 mRNA expression was 4.6-fold higher ($p<0.05$) in the taxane-resistant SKOV3TRip2 line compared to its parental cell line, SKOV3ip1.

**Downregulation of Jagged1 decreases viability and reverses taxane resistance in ovarian cancer cells in vitro.** To determine whether Jagged1 downregulation affects ovarian cancer cell viability *in vitro*, IGROV-AF1 and SKOV3TRip2 cells were transiently transfected with two different siRNA constructs against Jagged1 (JAG1_A or JAG1_B siRNA). JAG1_B siRNA decreased Jagged1 expression to a greater extent than JAG1_A siRNA in both IGROV-AF1 and SKOV3TRip2 cells, as determined by Western blot (Figure 2A). Concordantly, JAG1_B siRNA had the greatest effect on cell viability for both IGROV-AF1 (37.7% reduction, $p<0.05$) and SKOV3TRip2 (71.5% reduction, $p<0.001$) cells (Figure 2B, data points on y-axis). Given the increased expression of Jagged1 in the taxane-resistant SKOV3TRip2 line, we asked whether downregulation of
Jagged1 could sensitize resistant cells to chemotherapy. IGROV-AF1 and SKOV3TRip2 cells were transiently transfected with Jagged1-targeting siRNAs or control siRNA for 24 hours and exposed to increasing concentrations of docetaxel. Cell viability was assessed by MTT assay 4 days after the addition of docetaxel. As shown in Figure 2B, downregulation of Jagged1 did not increase the sensitivity of IGROV-AF1 cells to docetaxel (IC50 ~ 2 nM); however, downregulation of Jagged1 reduced the docetaxel IC50 from 40 nM to 10.2 nM in SKOV3TRip2 cells. Overcoming taxane resistance in the SKOV3TRip2 line but not in the already-sensitive IGROV-AF1 line suggests that the mechanism by which Jagged1 contributes to taxane resistance are the same as those responsible for acquired taxane resistance, rather than an additive effect that might be seen in any cell line. Tests for synergy indicate a CI = 0.77, suggesting moderate synergy in the SKOV3TRip2 line. To determine the mechanism by which Jagged1 downregulation may affect cell growth, cell cycle analysis was performed in a separate experiment. SKOV3TRip2 cells were exposed to control or anti-Jagged1 siRNA for 24 hours, followed by vehicle or docetaxel at IC50 levels for another 48 hours. Jagged1 downregulation alone and in combination with docetaxel induced a small but statistically significant increase in apoptosis (p<0.001, compared to control siRNA, p<0.01 compared to docetaxel alone) and an accumulation of SKOV3TRip2 cells in the G2/M phase (p<0.001, compared to control siRNA) (Figure 2C). Induction of apoptosis was confirmed by the presence of PARP cleavage in SKOV3TRip2 cells exposed to Jagged1 siRNA (Figure 2D). Jagged1 siRNAs had no sensitizing effect to carboplatin in SKOV3TRip2 and A2780cp20 cells (data not shown), and had no significant effect on the viability of Jagged1-negative A2780ip2 cells (Figure 2E).
**Human versus murine in vivo downregulation of Jagged1.** We have previously demonstrated that Jagged1 and other Notch family members are overexpressed in tumor-associated endothelial cells, and that Jagged1 downregulation prevented tube formation of HUVEC cells *in vitro* (11). Given the effects of Jagged1 downregulation on tumor cells as well, we sought to determine the relative effects of targeting Jagged in the stromal and malignant cell compartments individually. Additionally, inhibition of both compartments would simulate effects that would be expected in patients. There are no known inhibitors of Jagged1 for *in vivo* studies. Therefore we utilized a method for delivery of siRNA *in vivo* using chitosan (CH) nanoparticles. siRNA holds the additional advantage of inhibiting both Notch-dependent and Notch-independent effects mediated by Jagged1 through bidirectional signaling. We have previously demonstrated that twice-weekly administration of siRNA incorporated into biodegradable chitosan nanoparticles results in siRNA delivery to the tumor parenchyma with subsequent target downregulation in both stromal and malignant tumor cells (24-25, 28-29). In this study, nude mice were injected intraperitoneally with either SKOV3TRip2 or IGROV-AF1 cells and randomized to four treatment groups: 1) control siRNA; 2) murine-specific anti-Jagged1 siRNA; c) human-specific anti-Jagged1 siRNA; or d) both species-specific constructs. The specificity of each Jagged1 siRNA construct against the human or murine species was first demonstrated *in vitro* using human SKOV3TRip2 and murine MOEC cell lines by Western blot analysis (Figure 3A). For *in vivo* studies, siRNAs were incorporated into chitosan nanoparticles and administered IV twice per week. After four weeks of treatment, all mice were sacrificed and total tumor weight recorded. In
SKOV3TRip2 xenografts (Figure 3B), there was a reduction in tumor growth with anti-murine Jagged1 siRNA-CH (41.7%, p=0.089) compared to control siRNA, and human-specific anti-Jagged1 siRNA-CH significantly reduced tumor weight by 58.3% (p=0.042). The combination of both species-specific constructs resulted in significantly reduced tumor weight, by 87.5% compared to control siRNA (p=0.019). This represented an 82.1% reduction compared to murine siRNA-CH alone (p=0.13) and a 70% reduction compared to human siRNA-CH alone (p=0.03). Similarly, in IGROV-AF1 xenografts (Figure 3C), there was a moderate reduction in tumor weight with just murine-specific anti-Jagged1 siRNA-CH (48.8%, p=0.24) or human-specific anti-Jagged1 siRNA-CH (54.4%, p=0.27) compared to control siRNA. However, treatment with combined species-specific anti-Jagged1 siRNAs-CH again resulted in a significant decrease in tumor weight, by 93.1% compared to control siRNA-CH (p=0.008), by 84.9% compared to anti-human Jagged1 siRNA-CH alone (p=0.0046), and by 86.5% compared to anti-murine Jagged1 alone (p=0.012). There was not a statistically significant reduction in the number of nodules in each group, suggesting that the primary mechanism of reduced tumor size is reduced growth, rather than implantation and uptake of the tumor implants.

Given the Notch pathway’s implication in angiogenesis, immunohistochemical analysis of CD31 was performed to evaluate microvessel density to determine whether species-specific targeting of Jagged1 has anti-angiogenic effects. As shown in representative sections in Figure 3D, microvessel density was significantly reduced in SKOV3TRip2 tumors treated with anti-murine Jagged1 siRNA-CH (alone or in combination with anti-human Jagged1 siRNA-CH) compared to those treated with
control siRNA (from 21.2 to 8.7 vessels per hpf, Figure 3E, p<0.01). Anti-human Jagged1 siRNA-CH alone had no significant effect on microvessel density.

**Taxane sensitization with dual-compartment Jagged1 downregulation in vivo.** To determine the effect of Jagged1 downregulation on taxane sensitivity in vivo, nude mice were injected intraperitoneally with SKOV3TRip2 cells and randomized to four treatment groups (to begin 1 week after cell injection): 1) control siRNA-CH; 2) control siRNA-CH + docetaxel; 3) combined human and mouse-specific anti-Jagged1 siRNAs-CH; or 4) combined human and murine-specific anti-Jagged1 siRNAs-CH + docetaxel. After five weeks of treatment, mice were sacrificed and total tumor weight recorded. As shown in Figure 4A, control siRNA-CH + docetaxel treatment had no effect on SKOV3TRip2 tumor weight. In contrast, treatment with combined human and mouse-specific anti-Jagged1 siRNAs-CH significantly reduced tumor weight (by 78.4%) compared to control siRNA-CH + docetaxel (p=0.038). In addition, the combination of species-specific anti-Jagged1 siRNAs-CH and docetaxel further decreased tumor weight, by 94.8% compared to control siRNA-CH + docetaxel (p=0.017) and by 76.0% compared to dual-compartment Jagged1 downregulation alone (p=0.04).

To determine whether decreases in SKOV3TRip2 tumor weights following Jagged1 downregulation (alone and in combination with docetaxel) were due to decreased cell proliferation, apoptosis, or both, immunohistochemical analysis of PCNA and TUNEL staining were performed. Proliferation rates were significantly decreased in tumors treated with anti-human Jagged1 siRNA-CH, whether anti-human siRNA was used alone, in combination with anti-murine Jagged1 siRNA-CH, or in combination with
anti-murine Jagged1 siRNA-CH plus docetaxel (23.3%, 11.4% and 5.8%, respectively) compared to both control siRNA-CH (72.5%) or control siRNA-CH plus docetaxel (69.4%, p<0.001) (Figure 4B, C). Rates of apoptosis were not significantly different between the treatment groups, ranging from 3.9 to 6.6% (data not shown). The small percentage of cells undergoing apoptosis following Jagged1 downregulation (both in vitro and in vivo) suggest that decreased cell proliferation, rather than apoptosis, largely contributes to Jagged1 knockdown effects.

To determine whether increased taxane sensitivity observed with combined species-specific anti-Jagged1 siRNAs is due to effects on tumor cells, murine stromal cells, or both,, SKOV3TRip2 xenografts were treated with docetaxel plus control siRNA-CH, anti-human Jagged1 siRNA-CH, anti-murine Jagged1 siRNA-CH or combined anti-human/anti-murine Jagged1 siRNA-CH. As shown in Figure 4D, anti-murine Jagged1 siRNA-CH plus docetaxel had no effect on SKOV3TRip2 tumor weight compared to control siRNA-CH plus docetaxel. In contrast, treatment with either anti-human Jagged1 siRNA-CH plus docetaxel or anti-human/murine Jagged1 siRNA-CH plus docetaxel significantly decreased tumor weight (by 79.8%; p=0.001 and 74.6%; p=0.003, respectively). These data would suggest that unlike its role in angiogenesis, Jagged1’s role in taxane resistance is a characteristic of tumor cells, rather than transmitted through signals from the tumor stroma.

**Jagged1 downregulation contributes to decreased ovarian cancer cell viability in part through downregulation of Gli2.** In order to confirm that Jagged1 targeting was working through the Notch pathway, we evaluated mRNA levels of the Hes1 and Hey1
transcription factors, known downstream mediators of Notch signaling, by quantitative PCR. Surprisingly, downregulation of Jagged1 resulted in a modest increase in both HES1 (1.7-fold, p=0.27) and HEY1 (1.8-fold, p=0.22), rather than a decrease that would be expected from targeting Notch signaling (Figure 5A). Therefore, alternate pathways of effect were sought. The primary mediator of taxane resistance in general, and in the SKOV3TRip2 cell line specifically, is expression of MDR1, with 110-fold increased expression compared to parental SKOV3ip1 (30). Therefore we first examined whether Jagged1 downregulation reduced MDR1 expression. Paradoxically, decreasing Jagged1 with transient siRNA actually led to a nearly-significant 1.75-fold increase (p=0.06) in MDR1 expression (Figure 5B). To explore other potential mechanisms, we examined the Hedgehog signaling pathway (in particular, the downstream effectors GLI1 and GLI2) because of its involvement in stem cell maintenance, similar to the Notch pathway. Previous investigators have noted links between stem cell signaling pathways, such as the Notch and Wnt pathways (31) and the Notch and Hedgehog pathways (32). Expression of GLI1 was not significantly affected by Jagged1 siRNA compared to control siRNA; however, GLI2 expression was significantly reduced (by 2.20-fold, p=0.0016) following Jagged1 downregulation (Figure 5B). In addition, it was found that JAG1 and GLI2 (but not GLI1) gene expression levels significantly correlated among the ovarian cancer cell lines examined in this study (r=0.81, p=0.0273).

To further explore this potentially unique relationship between Jagged1 and the hedgehog transcription factor Gli2, we first examined whether this mechanism was Notch-dependent, since downregulation of Jagged1 appeared to have no effect on Notch downstream targets. To this end, we knocked down expression of Notch1 and Notch3,
key Notch receptors implicated in stem cell maintenance and ovarian cancer (5, 33), and examined \textit{JAG1} and \textit{GLI2} gene expression. As shown in Figure 5C and D, downregulation of either Notch1 or Notch3 had no effect on \textit{JAG1} or \textit{GLI2} expression, suggesting that Jagged1 influences Gli2 in a Notch-independent fashion. To examine the downstream effects of reduced \textit{GLI2} expression, we selectively targeted Gli2 using 2 different siRNA constructs (Figure 5E). First, we examined effects of apoptosis with Gli2 knockdown, and noted significant induction of PARP cleavage compared to control siRNA (Figure 5E). Interestingly, we found that Gli2 downregulation in turn significantly decreased \textit{JAG1} expression (1.64-fold, \( p=0.0028 \); Figure 5F), further suggesting a link between these signaling peptides. To determine if Gli2 plays a role in Jagged1-mediated taxane sensitization, SKOV3TRip2 cells were transiently transfected with Gli2-targeting siRNAs or control siRNA for 24 hours before the addition of increasing concentrations of docetaxel. Gli2 siRNAs alone reduced cell viability by up to 68\% (Figure 5G, \( p<0.001 \)), but reduced the docetaxel IC50 only slightly, from 40 nM to 29 nM. Because the taxane sensitizing effects of Gli2 targeting are not as pronounced as Jagged1 targeting alone, it appears that both Gli2 and additional Notch-independent pathways are at work in taxane resistance.
DISCUSSION

In the current study, we found that targeting Jagged1 in tumor cells induces apoptosis, reduces cell viability, and reverses taxane resistance in ovarian cancer cells both \textit{in vitro} and \textit{in vivo}, at least in part through downregulation of the Hedgehog mediator \textit{GLI2}. Additionally, knockdown of Jagged1 in tumor stromal cells reduces tumor growth through an anti-angiogenic mechanism. The participation of Jagged1 in both stromal and malignant cell compartments makes it an attractive target for therapy, and demonstrates the utility of a model whereby these compartments can be targeted independently to delineate the various contributions of different cells in the tumor microenvironment.

Previous studies have demonstrated aberrant expression of the Notch pathway in ovarian cancer (4-7). In particular, Jagged1 was found to be the primary Notch ligand expressed in ovarian cancer cells compared to Jagged2, DLL1, 3 and 4 (6). Jagged1 was also found to be overexpressed in endothelial cells purified from ovarian cancers compared to normal ovaries (11). Taken together, these studies indicate that Jagged1 would be a desirable therapeutic target in ovarian cancer, both from an anti-tumor and anti-angiogenic standpoint. In our study, we found that downregulation of Jagged1 resulted in decreased ovarian cancer cell viability \textit{in vitro}, most likely mediated through reduced cell proliferation and, to a lesser extent, induction of apoptosis. Our study is the first to demonstrate that targeting Jagged1 diminishes tumor burden \textit{in vivo}. Because there are no known inhibitors of Jagged1, we used chitosan nanoparticles to deliver Jagged1 siRNAs in tumor-bearing mice. These positively charged nanoparticles allow for the transport of siRNA across cellular membranes and are biodegradable,
biocompatible and have low immunogenicity (34-35). Targeting Jagged1 using this delivery system may also avoid the dose-limiting toxicities inherent to systemic Notch inhibitors such as gamma-secretase inhibitors (GSIs) (36). Selective targeting of Jagged1 using chitosan greatly decreased tumor burden and increased taxane sensitivity in orthotopic ovarian cancer mouse models. These results, combined with our observations in vitro, indicate that Jagged1 plays an important role in ovarian cancer cell survival. Whether these effects occur entirely through Notch signaling remains an open question. It has been suggested by Choi et al. (6) and others (15-16) that Jagged1 may have its own signaling function that is independent of the canonical Notch pathway. Indeed, the lack of a decrease in the expression of Notch downstream targets, *HES1* and *HEY1*, following Jagged1 downregulation in our study supports this mechanism. This potentially unique Notch-independent function of Jagged1 in human cancers, however, has yet to be fully explored.

The interaction between cancer cells and the surrounding stroma is increasingly becoming a focus of study in cancer research due to its role in tumor progression. This tumor-associated stroma is composed primarily of endothelial cells, which are necessary for tumor angiogenesis, and fibroblasts, which can secrete growth factors to the adjacent cancer cells. Recent reports suggest that Notch signaling can occur between tumor and stromal cells in some malignancies (37-38), indicating that targeting the Notch-ligand interaction in endothelial cells can have therapeutic applications. In addition, studies have shown that Jagged1 expression is crucial for normal vascular development during embryogenesis and that mutations of the *JAGGED1* gene can cause Alagille syndrome, a disease characterized by, among other deformities, congenital heart defects (39-40).
our study, we found that selectively targeting Jagged1 in the tumor stroma significantly reduced microvessel density (as measured by CD31) and, when combined with Jagged1 antagonism in cancer cells, the overall anti-tumor effect was greater than either anti-Jagged1 method alone. These data suggest that, unlike most cancer-associated targets which are expressed in only one compartment of the tumor, inhibiting Jagged1 activity could be used to target both the tumor and its developing vasculature, thereby having a potentially greater therapeutic benefit.

Chemoresistance remains a persistent obstacle in the treatment of ovarian cancer. While the clinical behavior of ovarian cancer suggests that most cancer cells are initially sensitive to chemotherapy, they subsequently either develop resistance or contain a population of cells that are inherently resistant. The latter hypothesis is consistent with what has become known as cancer stem cells or cancer initiating cells (CICs). These CICs are commonly believed to have enhanced tumorigenicity, differentiation capacity and resistance to chemotherapy in comparison to non-CICs. It is because of these features that CICs have been examined for molecular pathways and markers that could be targeted for therapeutic purposes. Recent studies have shown that the ancient developmental pathways Hedgehog, Wnt and Notch play important roles in the maintenance of CICs and that inhibiting these pathways may provide enhanced chemosensitivity when combined with traditional chemotherapies (8, 41-43). In our study, we sought to determine the mechanism whereby Jagged1, a known target of Wnt/β-catenin signaling (44-45) and a Notch ligand, might sensitize ovarian cancer cells to docetaxel. We chose to focus on the hedgehog pathway due to its involvement in CIC maintenance and multi-drug resistance (46-49). Interestingly, expression of GLI2, a
hedgehog transcriptional effector, was significantly decreased following Jagged1 knockdown, whereas expression of GLI1 and MDR1 was not. This relationship, one that appears to be Notch-independent, between Jagged1 and Gli2 was also found to work both ways, as knockdown of Gli2 diminished Jagged1 expression. Moreover, selective targeting of Gli2 using siRNA constructs decreased viability and increased sensitivity of ovarian cancer cells to docetaxel, although to a lesser degree than Jagged1 knockdown. These results suggest that inhibition of Gli2 contributes to the cell death and chemosensitization resulting from Jagged1 knockdown with other, as yet undefined, mechanisms likely playing a role as well. This connection between Jagged1 and Gli2 has not been previously identified and may have important therapeutic implications since targeting both Notch and Hedgehog, especially in combination with chemotherapy, is increasingly being advocated for the treatment of a variety of malignancies (3, 8, 50).

Collectively, the data presented in this study demonstrate that the Notch ligand Jagged1 contributes to taxane resistance, and targeting Jagged1 in ovarian cancer cells as well as in surrounding stroma significantly reduces growth through anti-proliferative, apoptotic, anti-angiogenic, and taxane-sensitizing effects. With the ability to identify subsets of cancer patients with Jagged1 overexpression, antagonism of this signaling molecule could ultimately provide a useful therapeutic strategy for ovarian cancer.
REFERENCES


FIGURE LEGENDS

**Figure 1. Jagged1 expression in ovarian cancer cell lines.**  A) mRNA expression of Jagged1 was quantified in IGROV-AF1 and 3 pairs of chemosensitive and chemoresistant ovarian cancer cell lines using quantitative PCR. Gene expression is shown as log2 transformed ΔCT values (difference between the CT value of the gene of interest (Jagged1) and that of the housekeeping gene (RPLP0)). *P < 0.01. B) Protein expression of Jagged1 was also measured using Western blot analysis. β-actin was used as a loading control. Blot is representative of 3 independent experiments.

**Figure 2. Downregulation of Jagged1 decreases viability and sensitizes to docetaxel in ovarian cancer cells.**  A) Downregulation of Jagged1 in IGROV-AF1 and SKOV3TRip2 cells using 2 different siRNA constructs was determined by Western blot analysis. B) IGROV-AF1 and SKOV3TRip2 cells transiently transfected with anti-Jagged1 siRNAs had decreased viability; SKOV3TRip2 cells were sensitized to docetaxel (Doc) following Jagged1 knockdown. C) Cell cycle analysis demonstrated that Jagged1 downregulation (alone and in combination with docetaxel) induces an accumulation of SKOV3TRip2 cells in the sub-G0 phase, indicative of cells undergoing apoptosis, and the G2/M phase. D) PARP cleavage, an indicator of apoptosis, was also observed in SKOV3TRip2 cells exposed to Jagged1 siRNA (alone or in combination with docetaxel), as determined by Western blot analysis. Blots are representative of 3 independent experiments. E) Jagged1 siRNAs had no effect on the viability of A2780ip2 cells, which do not express Jagged1.
Figure 3. Human versus murine in vivo downregulation of Jagged1.  A) Species-specific downregulation of Jagged1 in human SKOV3TRip2 and mouse MOEC cell lines was determined by Western blot analysis.  B) Mice injected intraperitoneally with SKOV3TRip2 cells were treated with siRNAs incorporated in chitosan (CH) nanoparticles.  Mice treated with the combination showed a significant reduction in tumor weight compared to treatment with control siRNA or either single species-specific Jagged1 siRNA.  C) Similarly, mice injected with IGROV-AF1 cells had significantly decreased tumor weight after treatment with combination murine- and human-Jagged1 siRNAs compared to control siRNA or either single species-specific siRNA.  Mean tumor weights with standard deviation are presented.  D) SKOV3TRip2 tumors were subjected to immunohistochemical analysis of CD31 to evaluate microvessel density.  Xenografts treated with anti-murine Jagged1 siRNA had significantly decreased microvessel density compared to those treated with control siRNA.  Representative histologic sections are shown for the various treatment groups (black bar = 100 µm), with mean and standard deviation values across each treatment group shown in the adjoining graph (E).  Cntrl = control; Hs=Human; Mm=Murine; siRNA-CH=siRNA in chitosan nanoparticles.  *P < 0.05.

Figure 4. Taxane sensitization with dual-compartment Jagged1 downregulation in vivo.  A) Mice injected intraperitoneally with SKOV3TRip2 cells were treated with either control siRNA, control siRNA + docetaxel, combined human- and murine-Jagged1 siRNAs or combined Jagged1 siRNAs + docetaxel.  All siRNA constructs were incorporated in chitosan nanoparticles.  Mice treated with the combination species-
specific Jagged1 siRNAs + docetaxel showed a significant reduction in tumor weight compared to treatment with either Jagged1 siRNAs or docetaxel alone. Mean tumor weights with standard deviation are presented. *P < 0.05. B) SKOV3TRip2 tumors were subjected to immunohistochemical analysis of PCNA to evaluate cell proliferation. Xenografts treated with anti-human Jagged1 siRNA or combined species-specific Jagged1 siRNAs (alone and in combination with docetaxel) had significantly decreased proliferation rates compared to those treated with control siRNA or control siRNA plus docetaxel. Representative histologic sections are shown (B) for the various treatment groups (black bar = 100 µm), with mean and standard deviation values across each treatment group shown in the adjoining graph (C). *P<0.05, **P < 0.001. D) Mice injected intraperitoneally with SKOV3TRip2 cells were treated with docetaxel plus either control siRNA, anti-human Jagged1 siRNA, anti-murine Jagged1 siRNA or combined human- and murine-Jagged1 siRNAs. All siRNA constructs were incorporated in chitosan nanoparticles. Mice treated with anti-human and not anti-mouse Jagged1 siRNA showed a significant reduction in tumor weight in combination with docetaxel. Mean tumor weights with standard deviation are presented. *P < 0.01.

Figure 5. Gli2 reduction following Jagged1 downregulation contributes to decreased ovarian cancer cell viability and taxane-sensitization. SKOV3TRip2 cells were transiently transfected with control siRNA or anti-Jagged1 siRNA for 72 hours and examined for gene expression using quantitative PCR. (A) Notch signaling targets HES1 and HEY1 were moderately increased as a result of Jagged1 downregulation. Error bars represent SEM. (B) Expression of GLI2, but not MDR1 or GLI1, was significantly
decreased following Jagged1 downregulation. C) Knockdown of Notch1 did not affect $JAG1$ or $GLI2$ expression. D) Knockdown of Notch3 also had no effect on $JAG1$ or $GLI2$ expression. E) PARP cleavage was observed in SKOV3TRip2 cells transfected with Gli2 siRNAs, as determined by Western blot (representative of 3 independent experiments). F) Expression of $JAG1$ was significantly decreased following Gli2 downregulation. G) Knockdown of Gli2 using 2 different siRNA constructs decreased viability and increased sensitivity to docetaxel in SKOV3TRip2 cells. *$P < 0.01$. 
Figure 1

A

Jagged1 qPCR

Gene Expression ($2^{∆∆Ct}$)

A2780ip2  A2780cp20  HeyA8  HeyA8MDR  IGROV-AF1  SKOV3ip1  SKOV3TRip2

*B<0.01

B

Jagged1  β-actin

A2780ip2  A2780cp20  HeyA8  HeyA8MDR  IGROV-AF1  SKOV3ip1  SKOV3TRip2
Figure 2

**A**

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<th>SKOV3TRip2</th>
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<td>Jagged1</td>
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<tr>
<td>β-Actin</td>
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**B**

**C**

Cell cycle analysis with Jagged1 siRNA and docetaxel

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<td>24.0</td>
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**D**

IGROV-AF1

- Control siRNA
- JAG1_A siRNA
- JAG1_B siRNA

Full-length PARP
- Control siRNA
- JAG1_B siRNA

Cleaved PARP
- Control siRNA
- JAG1_B siRNA

β-actin
- Control siRNA
- JAG1_B siRNA

**E**

A2780ip2

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Figure 3

A

SKOV3TRip2

MOEC

Jagged1

β-Actin

B

SKOV3TRip2

C

IGROV-AF1

Tumor Weight (g)

*P<0.05

D

Control siRNA-CH

Hs Jag1 siRNA-CH

Mm Jag1 siRNA-CH

Mm + Hs Jag1 siRNA-CH

E

Microvessel density with anti-Jagged1 siRNA

Mean vessels per hpf

*P<0.01

21.2

19.3

8.7

9.1
Figure 4

**A**

SKOV3TRip2

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<td>Control siRNA-CH + Doc</td>
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<tr>
<td>Hs + Mm Jag1 siRNA-CH</td>
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<tr>
<td>Hs + Mm Jag1 siRNA-CH + Doc</td>
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*P<0.05

**B**

Control siRNA-CH

Control siRNA-CH + Doc

Mm Jag1 siRNA-CH

Hs Jag1 siRNA-CH

**C**

Proliferation index with anti-Jagged1 siRNA

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<td>Hs + Mm Jag1 siRNA-CH</td>
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<tr>
<td>Hs + Mm Jag1 siRNA-CH + Doc</td>
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**P<0.001

**D**

SKOV3TRip2

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<tr>
<td>Hs Jag1 siRNA-CH + Doc</td>
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<tr>
<td>Mm Jag1 siRNA-CH + Doc</td>
<td>0.017</td>
</tr>
<tr>
<td>Hs + Mm Jag1 siRNA-CH + Doc</td>
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*P<0.01
Figure 5

A  Effect of Jag1 downregulation on Hes1 and Hey1

B  Effect of Jag1 downregulation on MDR1, Gli1 and Gli2

C  Effect of Notch1 downregulation on Jag1 and Gli2

D  Effect of Notch3 downregulation on Jag1 and Gli2
Figure 5

E

F

Effect of Gli2 downregulation on Jag1

Control siRNA
GLI2_A siRNA
GLI2_B siRNA

Gli2
Full-length PARP
Cleaved PARP
β-actin

GLI2
JAG1

Relative Expression

Control siRNA
GLI2_B siRNA

*P<0.01

G

OD 450

0.0 1.0 10.0 100.0 1000.0

0.0 0.2 0.4 0.6 0.8 1.0 1.2

Docetaxel (nM)
Targeting the Notch Ligand Jagged1 in Both Tumor Cells and Stroma in Ovarian Cancer

Adam D Steg, Ashwini A Katre, Blake W. Goodman, et al.

*Clin Cancer Res* Published OnlineFirst July 13, 2011.

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