Notch3 Overexpression Promotes Anoikis Resistance in Epithelial Ovarian Cancer via Upregulation of COL4A2

Caitlin W. Brown1, Alexander S. Brodsky2, and Richard N. Freiman1,2

Abstract

Ovarian cancer is a lethal disease with the majority of diagnosed women having distant metastases. Interestingly, although Notch3 overexpression has been correlated with poor survival in epithelial ovarian cancer (EOC), little is known about its mechanism of action. Data show that Notch3 specifically promotes anoikis resistance. In addition, data indicate a positive role for focal adhesion kinase (FAK) as well as downstream signaling kinases such as Akt and Erk 1/2 in promoting anchorage-independent growth. Mechanistically, both mRNA transcript and protein levels of type IV collagen (COL4A2) are reduced when Notch3 levels are decreased and exogenous collagen IV supplementation reverses the anoikis sensitivity. Reduction of COL4A2 expression by RNAi-mediated knockdown induces cell death. Finally, elevated Notch3 expression levels correlate with higher COL4A2 expression in human ovarian tumor specimens.

Implications: These data highlight type IV collagen as a novel therapeutic target for metastatic EOC.

Visual Overview: http://mcr.aacrjournals.org/content/early/2014/11/25/1541-7786.MCR-14-0334/F1.large.jpg

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Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecologic malignancy. In 2011, the National Cancer Institute estimated that 21,990 women would be diagnosed with ovarian cancer and 15,460 women would die from it. Approximately 70% of women diagnosed with the disease will have metastases beyond the regional lymph nodes. Once the cancer has spread from lymph nodes, the 5-year survival rate drops from 72% to 27% (1). Current research focuses on developing new targets to improve overall survival (OS) as well as quality of life. The Notch signaling pathway has lately come into focus as a potential target for ovarian cancer treatments (2). Notch3 overexpression in human ovarian tumor samples correlates with shorter progression-free survival as well as shorter OS (3). Moreover, in vitro data show that Notch3 signaling protects cells from apoptosis (4, 5). Expression of Notch3 can be oncogenic or tumor suppressive depending on its mechanism of action. Data show that Notch3 specifically promotes anoikis resistance in EOC cells through the excess expression of ECM proteins. These overexpressed ECM types, it not well understood in the context of EOC. Proteins such as GLI1, c-Met, and HTRA1 have been implicated in anoikis resistance to successfully survive in nonadherent conditions. Several mechanisms of anoikis resistance are known, including the overexpression of ECM proteins. These overexpressed ECM components coat the cell surface and allow them to “carry their own soil” in a nonadherent environment, thus maintaining downstream prosurvival signals. Classic prosurvival proteins like Akt and Erk 1/2 have been shown to cross-talk downstream of FAK to promote nonadherent cell survival (6, 9). Integrin and EGFR signaling have also been implicated in regulating anoikis resistance (10). Although anoikis resistance has been studied in many cancer types, it is not well understood in the context of EOC. Proteins such as GLI1, c-Met, and HTRA1 have been implicated in anoikis resistance in EOC (9, 11, 12). Recently, Notch3 overexpression has been implicated in preventing apoptosis in EOC cells (6). Here, we provide evidence that elevated Notch3 levels promote anoikis resistance in EOC cells through the excess expression of the collagen type IV alpha 2 (COL4A2) gene. Elevated Notch3 levels correlate with prosurvival signaling via FAK and activated Akt and Erk 1/2 to repress the proapoptotic protein Bim. When Notch3 and COL4A2 are reduced in ovarian cancer cells, exogenous treatment with collagen IV is sufficient to restore cell survival signaling. Moreover, we uncovered a highly positive correlation between notch3 and col4a2 mRNA levels in human peritoneal cavity, EOC cells metastasize by breaking off and using the flow of peritoneal fluid to seed the abdomen. Anoikis is a process by which normal cells undergo a specific form of apoptosis following detachment from the extracellular matrix (ECM). Normal cells that remain in contact with the ECM cross-talk with the microenvironment, promoting prosurvival signaling. Such signaling is often integrated via focal adhesion kinase (FAK), a protein complex that connects interior and exterior cellular signaling. Lack of ECM contact disrupts these prosurvival signals, resulting in a specialized form of apoptosis termed anoikis. Ovarian cancer cells must develop anoikis resistance to successfully survive in nonadherent conditions.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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ovarian metastases compared with levels in primary ovarian tumors. Together, these data illuminate a Notch3–COL4A2 circuit promoting ovarian cancer cells’ ability to resist anoikis and progress to metastases in vitro and in patients.

**Materials and Methods**

**Cell lines and reagents**

Human ovarian cancer cell lines ES2, Hey, OVCAR-8, and SKOV-3 were a gift from Dr. Alexander Brodsky (Brown University, Providence, RI). IGROV-1, OVCAR-3, and OVCAR-4 were obtained from the National Cancer Institute. A2780 cells were purchased from Sigma (93112519). Fallopian tube secretory cells (FTSEC240) were generously provided by the Drapkin laboratory at the Dana Farber Cancer Institute (Boston, MA) and maintained as described previously (13). All cells were maintained at 37°C in 5% CO2. ES2, Hey, OVCAR-8, and SKOV-3 cell lines were maintained in DMEM (Sigma) in 1% penicillin/streptomycin and 10% heat-inactivated FBS. IGROV-1, OVCAR-3, and OVCAR-4 cell lines were maintained in RPMI-1640 (HyClone), 1% penicillin streptomycin, and 10% heat-inactivated FBS (Thermo Fisher Scientific). Z-VAD–FMK was purchased from R&D Systems. Y11 was purchased from Tocris (4498). Antibodies are detailed in Supplementary Table S1. siRNA and transfection reagents were purchased from ThermoScientific. On-TargetPlus Nontargeting control (D-001810-01-05), On-TargetPlus SmartPool Notch3 (011093) and On-TargetPlus SmartPool COL4A2 (003645), DharmaFECT-1 (T-2001-01). Human collagens type I and IV were purchased from Millipore (CC050 and CC076, respectively).

**Cell line screen**

Cells from all eight lines were collected and screened for Notch3 expression levels by qPCR and Western blotting as described previously (14). Primer sequences are detailed in Supplementary Table S2. Cells were transfected according to the manufacturer’s protocol with DharmaFECT-1. All cells were collected 48 hours posttransfection unless otherwise noted.

**Cell viability assays**

Cells were plated to 50% confluence, transfected with siRNA 24 hours after plating and harvested 48 hours posttransfection by the standard trypsinization protocol and stained with trypan blue as described previously (15). Z-VAD–FMK was added at 50 μmol/L and cells were treated concurrently with siRNA for 48 hours. Cells used in adherence assays were transfected 24 hours before trypsinization and replated in adherent (A) or nonadherent (NA) dishes. Y11 was used at 10 μmol/L and cells were treated after replating on to A or NA dishes. LY294002 was added at 20 μmol/L and cells were treated 2 hours before collection.

**Collagen rescue assay**

Twenty-four hours posttransfection, siControl or siNotch3 cells were plated on dishes precoated with PBS, collagen type I or type IV at 6 μg/cm². Then, 24 hours after plating, cells were collected for counting. Coll4a2 collagen rescue experiments were conducted similarly.

**Primary and metastatic human tumor samples**

Pearson correlations and their significance were calculated in Microsoft Excel. Primary and metastatic tumor data are available at GSE30587. All TCGA (The Cancer Genome Atlas) data were downloaded from the TCGA data portal using the published dataset freeze (http://www.nature.com/nature/journal/v474/n7353/full/nature10166.html). All TCGA data include primary ovarian tumors only. The Australian Oncology Group microarray data for ovarian tumors, GSE9891, were downloaded from GEO and processed using RMA. Q values were calculated in R.
Statistical analysis

Two-way ANOVA and the Tukey test were used and a P value of 0.05 was considered significant. All data are reported as means ± SD. Statistical analyses were performed using GraphPad software.

Results

Notch3 signaling promotes anoikis resistance in human ovarian cancer cell lines

The Notch signaling pathway has emerged as a therapeutic target in many diverse types of cancer (16–18). In EOC, oncogenic Notch3 signaling has been shown to protect human ovarian cancer cells from apoptosis (6, 19). We hypothesized that an elevated level of Notch3 was not only protecting these cells from classical cell death but that Notch3 was preventing a specific type of apoptosis: anoikis. To test this hypothesis, we screened five human EOC cell lines and an immortalized, nontransformed fallopian tube secretory cell line (FTSEC) for relative levels of Notch3 expression (Fig. 1A–C) and established a reliable siRNA-mediated knockdown of Notch3 in a subset of these cells using a pool of four siRNAs (Fig. 1D and E). The specificity and potency of individual siRNAs were tested by qPCR and found to be slightly less effective but equally specific to the siRNA pool (Fig. 1F). We found that of five cell lines examined, three had significantly increased levels of Notch3 at the mRNA and protein levels: A2780, OVCAR-3, and IGROV-1. To examine the cellular response to a reduction of Notch3 signaling, we used a trypan blue exclusion assay to assess the extent of cell viability and potency of siRNA treatment. To this end, trypsinized IGROV-1 cells that had been treated with Notch3 siRNA for 24 hours were transferred to either adherent or ultra-low attachment plates. At 48 hours posttransfection, we found that a reduction in Notch3 expression resulted in a significant increase in trypan blue–positive/dead cells (Fig. 2A). In addition, concurrent treatment with the pan-caspase inhibitor Z-VAD–FMK resulted in a rescue of the cell population at 48 hours posttransfection (Fig. 2B). To confirm that siNotch3 cells are undergoing apoptosis, we examined levels of cleaved PARP and saw that siNotch3 cells had elevated levels of cleaved PARP compared with siControl cells by immunoblot analysis (Fig. 2C). These data indicate that siNotch3 knockdown IGROV-1 cells were undergoing apoptosis, as had been previously reported in other cell lines (19).

To determine whether siNotch3 cells were undergoing anoikis, we compared their survival in adherent and nonadherent conditions. To this end, trypsinized IGROV-1 cells that had been treated with Notch3 siRNA for 24 hours were transferred to either adherent or ultra-low attachment plates. At 48 hours posttransfection, we used trypan blue exclusion to assess the extent of cell viability. We found that there was significantly more dead cells in the siNotch3 cells cultured in nonadherent conditions than in control cells in nonadherent wells or Notch3-knockdown cells in adherent conditions (Fig. 3A). A nonsignificant increase in trypan blue–positive cells was seen in nonadherent siControl cells as compared with adherent siControl cells. A small increase in cell death was expected and observed in nonadherent siControl cells following transfer to nonadherent conditions. To support the notion that these cells were undergoing anoikis and not just apoptosis, we used the FAK inhibitor Y11 with siControl and siNotch3 cells. This inhibitor prevents FAK from autophosphorylation at Y397 and activating downstream prosurvival pathways. Treatment with 10 μmol/L Y11 increased trypan blue–positive cells in nonadherent siControl cells but had no significant impact on siNotch3 cells in either adherent or nonadherent conditions (Fig. 3B).

We also observed that the levels of phosphorylated FAK at Y397 were reduced in the siNotch3 cells as compared with siControl (Fig. 3C). One of the hallmarks of anoikis is the upregulation of the proapoptotic protein Bim (19). Analysis by immunoblot showed that Bim was enriched in siNotch3 knockdown cells compared with siControl cells (Fig. 4A). Plating siNotch3 cells in nonadherent conditions further increased Bim expression and induced the expression of the L isoform of Bim in addition to the EL isoform. An increase in Bim in control cells in nonadherent conditions was expected because of the increase in cell death shown in Fig. 3A. Interestingly, mRNA levels of bim were not significantly altered (Fig. 4D). This result agrees with published data suggesting a posttranscriptional mechanism of Bim suppression (20). In addition, levels of other proapoptosis proteins Bad, Bax, and Bak were not significantly altered between siControl and siNotch3 cells.
Reduction of Notch3 expression did not significantly affect expression of the prosurvival proteins Bcl-2, Bcl-XL, or Mcl-1. Together, these data indicate that siNotch3 cells are undergoing intrinsic, Bim-mediated anoikis and that elevated levels of both these proteins (11, 20). We examined total and activated levels of pAkt, pErk 1/2, and concomitant increase in Bim in both phospho-Akt and phospho-Erk 1/2 (Fig. 5B), suggesting cross-talk between these pathways during Notch3-mediated anoikis resistance.

Notch3 signaling promotes the expression of the type IV alpha 2 collagen gene

One of the mechanisms by which cancer cells can induce anoikis resistance is via the overexpression of ECM proteins (21). We hypothesized that the anoikis resistance we observed in human ovarian cancer cells may be due to the cells coating themselves in ECM, activating integrins, and inducing prosurvival signaling in the absence of adherence to the basement membrane. Collagen type IV is a significant component of basement membranes. To determine whether collagen was being aberrantly expressed, we assessed levels of collagen alpha chains by quantitative RT-PCR. We found that mRNA levels of col4a2 were significantly decreased in siNotch3 cells compared with siControl in both IGROV-1 and OVCAR-3 cell lines (Fig. 6A and B). Reduction in Notch3 levels did not alter levels of col4a2 expression (Supplementary Fig. S1). As the promoters of col4a2 and col4a1 are in a head-to-head conformation, we examined expression levels of both col4a2 and col4a1. However, in OVCAR-3 siNotch3 cells, the decrease in both col4a2 and col4a1 was statistically significant (Fig. 6C). These data suggest that Notch3 promotes the proper expression of col4a2, which may promote anoikis resistance.

Given the key role type IV collagens have in the basement membrane and our finding that col4a2 expression was selectively reduced in siNotch3 cells, we aimed to determine whether siNotch3 cells could be rescued by the addition of exogenous type IV collagen. To that end, we coated plates with PBS, type I collagen or type IV collagen, on which we seeded cells that had been transferred with nontargeting control siRNA or Notch3 siRNA for 24 hours. We then determined the extent of cell death by trypan blue exclusion 24 hours after plating and found that type IV but not type I collagen significantly improved cell survival in siNotch3 samples (Fig. 6D). These data indicate that increased expression of type IV collagen may promote anoikis resistance.

To more definitively determine the role of col4a2 in the promotion of anoikis resistance in ovarian cancer, we transfected IGROV-1 cells with col4a2 siRNA. We used qPCR to measure the expression of col4a2 at the mRNA level and observed a decrease of col4a2 and a concurrent increase in col4a1 expression (Fig. 7A). Assessment of cell death by trypan blue exclusion revealed a significant increase in cell death among the siCol4a2 samples (Fig. 7B). Analysis of Bim, phospho-Akt, and phospho-Erk 1/2 in siCol4a2 samples phenocopied siNotch3 cells with a reduction in pAkt, pErk 1/2, and concomitant increase in Bim in both the IGROV-1 and A2780 cell lines (Fig. 7C). Similarly, siCol4a2 cells were rescued via plating on exogenous type IV collagen, as were the siNotch3 cells (Fig. 7D). A similar reduction in phosphorylated Akt and Erk 1/2 was seen in A2780 siCol4a2 cells. These
experimental data indicate that the Notch3–Col4α2 circuit promotes anoikis resistance in human ovarian cancer cells.

**Increased notch3 expression correlates with increased col4α2 mRNA expression in human ovarian tumors and their metastases**

To determine whether the relationship between notch3 and col4α2 observed in ovarian cancer cells is relevant to tumors derived from patients with EOC, we examined the correlation of mRNA expression using multiple datasets. Strikingly, the expression of notch3 and col4α2 are significantly correlated in a small but matching set of nine primary tumors and their corresponding metastatic tumors (Fig. 8). This relationship is also observed in large tumor datasets from TCGA (Supplementary Table S3). Together, these observations are consistent with the positive regulation of notch3 overexpression with col4α2 expression in human ovarian tumors and metastases. Together, the clinical and experimental data suggest that targeting the Notch3–COL4A2 circuit may have beneficial effects in controlling the development or spread of EOC.

**Discussion**

Like many advanced cancers, metastatic EOC remains difficult to treat. Standard therapies result in remission but rarely a cure. Survival data suggest that preventing or reversing anoikis resistance could assist in preventing tumor cell spread and increasing survival. The results of recent studies show that aberrant Notch3 signaling promotes survival in ovarian cancer cells (19, 22). We confirmed these results and demonstrated that not only does Notch3 prevent apoptosis, it prevents a very specific form of apoptosis: anoikis. We demonstrated that Notch3 promotes anoikis resistance by promoting the expression of col4α2 and therefore the completion of the type IV collagen trimer—a key component of basement membrane.

The increased expression of type IV collagen may allow advanced ovarian cancer cells to deceive proteins like integrins, which are charged with detection of ECM contact, into maintaining prosurvival signals even when no true ECM contact is at hand. Via these linker proteins, the activation of FAK and the activation of key prosurvival cytosolic proteins like Akt and Erk 1/2, the cell is able to repress Bim, which is responsible for the initiation of anoikis.

Indeed, this reduction in collagen IV expression is so important to cell survival that growth on exogenous collagen type IV is all that is required to rescue dying siNotch3 ovarian cancer cells. In addition, reduction in col4α2 expression induces cell death similarly to the reduction in Notch3 and prevents phosphorylation of...
Akt and Erk 1/2, while promoting the expression of Bim. This demonstrates a reliance of EOC cells on these external signals to maintain viability when nonadherent. Our data also show that mRNA expression of bim is not altered in siNotch3 cells. C, protein level expression of the col4a2 chain is reduced in IGROV-1-siNotch3 cells as compared with siControl cell by immunoblot analysis. D, exogenous collagen IV rescues Notch3 knockdown cells (**, P < 0.01; ***, P < 0.005). IGROV-1-cells 24 hours posttransfection with Notch3 siRNA show significantly less cell death when plated on exogenous human collagen type IV for 24 hours than siNotch3 cells plated in human collagen Type I or control wells (P < 0.01).

Notch3 overexpression has been demonstrated in vitro to prevent apoptosis, induce chemoresistance, and alter the cell cycle (5, 24). Altered copy number of NOTCH3 is associated with aggressive tumor behavior and a poor prognosis (2, 5). Our results indicate that Notch3 promotes cross-talk between the ECM and cytosolic proteins, playing a significant role in disease progression. Not only can the ECM provide prosurvival signals but also the collagen matrix can alter the cell cycle and induce proliferation changes (25), which may account for some of the cell-cycle alterations observed in previous studies. The ECM and collagen are increasingly understood to be important mediators of EOC progression. Transcriptional differences in collagen gene

Figure 6. Notch3 is required for proper expression of col4a2. Both IGROV-1 and OVCAR-3 siNotch3 cells express significantly less col4a2 mRNA (A and B) than their control counterparts (**, P < 0.01; ***, P < 0.005; ****, P < 0.001). Col4a2 mRNA expression is not significantly altered in siNotch1 cells. C, protein level expression of the col4a2 chain is reduced in IGROV-1-siNotch3 cells as compared with siControl cell by immunoblot analysis. D, exogenous collagen IV rescues Notch3 knockdown cells (**, P < 0.01; ***, P < 0.005). IGROV-1-cells 24 hours posttransfection with Notch3 siRNA show significantly less cell death when plated on exogenous human collagen type IV for 24 hours than siNotch3 cells plated in human collagen Type I or control wells (P < 0.01).

Figure 7. Collagen-type IVa2 is critical to ovarian cancer cell survival. A, reduction in Col4a2 expression induces cell death. IGROV-1 cells transfected with Col4a2 siRNA have significantly more cell death and fewer total cells compared with their control counterparts (***, P < 0.005). B, type IV collagen is sufficient to rescue siCol4a2 cells from cell death (**, P < 0.01). C, siCol4a2 shows a reduction in phosphorylated Akt and Erk 1/2 as well as an increase in Bim. Shown are representative samples of three biologic replicates. D, siCol4a2 cells plated on exogenous collagen type IV have significantly fewer dead cells and more total cells than siCol4a2 cells plated in control wells (**, P < 0.01).
expression have been associated with poor OS in metastatic serous ovarian cancer and type IV collagen expression has been shown to be higher in metastatic EOC tumors than in the primary tumor (26, 27). These data, combined with our results, indicate that more work is required on the interactions between the cell surface, the ECM and EOC disease progression.

In addition, although the majority of studies in human samples have been performed using high-grade serous tumors, some data suggest that endometrioid-type tumors may also develop deleterious Notch3 amplifications in recurrent tumors (19). The three cell lines used in our studies have been shown to be of different subtypes. Although OVCAR-3 cells are likely to be of the high-grade serous (HGSOC) subtype, the A2780 are not (28). IGROV-1 cells are considered “hypermutated” and may have developed new adaptations, although the original tumor was determined to be mainly endometrioid with some characteristics of HGSOC and undifferentiated foci (29).

Although the effect of Notch3 overexpression is increasingly studied in EOC, anoikis resistance remains relatively unexamined. However, we feel that exploring this concept in the context of EOC may offer new targets for metastatic tumors. For example, studies in colon cancer have shown that the expression of type IV collagen is the key to anoikis resistance (30). In EOC, downregulation of Htra1 was shown to promote anoikis resistance (31). Stress has also been shown to activate FAK and protect ovarian cancer cells from anoikis by hormonal regulation (32). The overexpression of TrkB or c-Met has also been implicated in the development or maintenance of anoikis resistance (11, 33). We have seen that the expression of type IV collagen is the key to anoikis resistance in three divergent models of EOC: IGROV-1, OVCAR-3, and A2780 cells. The stimulation of cell survival by the expression of the type IV collagen chain may activate different cytosolic prosurvival pathways, depending on the EOC subtype, stage of disease, and mutations acquired.

We did not directly examine binding of the Notch3/CSL complex to the col4a2 promoter, although this is a logical next step. Given the dramatic changes in expression of col4a2 mRNA following reduction of Notch3 expression, col4a2 could be a direct target of Notch3 signaling. However, the Notch pathways are notoriously complex and further analysis by ChIP or ChIP-seq is required to better understand the regulation of col4a2 by Notch3. It was also interesting to see that a reduction in Notch1 did not significantly alter the expression of col4a2. Because of the specificity of Notch signaling, it is not completely surprising that the different receptors would have unique downstream targets and affects. This difference only highlights the targeted nature of Notch signaling and the importance of understanding the impact of each isoform. In that vein, it could be possible for expression levels of Notch3 to affect signaling, that is, amount of Notch3 expressed could correlate with different cellular outcomes.

Chen and colleagues (24) showed in a ChIP-on-chip analysis of Notch3 targets in the OVCAR-3 cell line, two integrins were shown to be significantly enriched: ITGA2B and ITGAE. These could be noteworthy alterations in expression as both are involved in adhesion of leukocytes and have the RGD peptide sequences required to bind collagens (34). Though the binding of CD41 and CD103 to type IV collagen is not well characterized, it can be speculated that expression of these integrin alpha chains allowed the EOC cell to fine-tune the prosurvival signaling through FAK to promote survival in a specific set of environmental conditions.

Long-term survival for patients with advanced ovarian cancer is at best uncertain. Novel therapies are required to make significant gains in 5-year survival rates in these populations. Targeting the Notch3-specific pathway and its downstream effects have been shown to be promising—indeed there are clinical trials underway using Notch3 inhibitors in combination with standard chemotherapeutics. One of the main goals of these Notch3 inhibitors is the "resensitization" of tumors that have developed resistance to mainstream chemotherapeutics. We hope these mechanistic data will provide novel targets and strategies for future clinical trials and enhance the responsiveness of tumors to treatment.

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

Authors’ Contributions
Conception and design: C.W. Brown, R.N. Freiman
Development of methodology: C.W. Brown
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.W. Brown
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.W. Brown, A.S. Brodsky
Writing, review, and/or revision of the manuscript: C.W. Brown, A.S. Brodsky, R.N. Freiman
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.W. Brown
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![Figure 8](image-url)

**Figure 8.**
Notch3 overexpression correlates with increased col4a2 expression in metastatic versus primary human ovarian tumors. Pearson correlation of 0.77 indicates a strong correlation between notch3 expression and col4a2 expression (P < 0.000143).
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