Cancer Research

Notch Signaling in CD66$^+$ Cells Drives the Progression of Human Cervical Cancers

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Abstract

Human epithelial tumor progression and metastasis involve cellular invasion, dissemination in the vasculature, and regrowth at metastatic sites. Notch signaling has been implicated in metastatic progression but its roles have yet to be fully understood. Here we report the important role of Notch signaling in maintaining cells expressing the carcinoembryonic antigen cell adhesion molecule CEACAM (CD66), a known mediator of metastasis. CD66 and Notch1 were studied in clinical specimens and explants of human cervical cancer, including specimens grown in a pathophysiologically relevant murine model. Gene expression profiling of CD66$^+$ cells from primary tumors showed enhanced features of Notch signaling, metastasis, and stemness. Significant differences were also seen in invasion, colony formation, and tumor forming efficiency between CD66$^+$ and CD66$^-$ cancer cells. Notably, CD66$^+$ cells showed a marked sensitivity to a Notch small molecule inhibitor. In support of studies in established cell lines, we documented the emergence of a tumorigenic CD66$^+$ cell subset within a metastatic lesion-derived cervical-cancer cell line. Similar to primary cancers, CD66 expression in the cell line was blocked by chemical and genetic inhibitors of ligand-dependent nuclear Notch signaling. Collectively, our work on the oncogenic properties of CD66$^+$ cells in epithelial cancers provides insights into the nature of tumor progression and offers a mechanistic rationale to inhibit the Notch signaling pathway as a generalized therapeutic strategy to treat metastatic cancers. *Cancer Res; 71(14); 4888–97. ©2011 AACR.*

Introduction

Notch signaling is an evolutionarily conserved pathway that regulates cell-fate determination, differentiation and self-renewal (1). The core Notch pathway comprises transmembrane receptors that are cleaved by γ-secretase on binding to the Delta, Serrate, Lag-2 (DSL) family of ligands. The cleaved product localizes to the nucleus and regulates transcription through intermediates such as CBF1, Su(H), and Lag1 (CSL). Truncated alleles of *Notch* generate constitutively active proteins that can drive oncogenesis. For example, such alleles are known to cooperate with both adenoviral (2) and Human Papillomavirus (HPV) oncogenes (3) in transforming epithelial cells. These prooncogenic effects of Notch signaling are mediated by activation of prosurvival and proliferation pathways such as PI3K/AKT (3), NFκB, and Ras/MAPK (4). Consistent with these observations, many human epithelial cancers, including that of the cervix, show enhanced expression of Notch and its ligands (4–6) along with features of activated NFκB and PI3K/AKT (7). There are no reported mutagenic events that might lead to sustained ligand-independent Notch activation in human epithelial cancers. However, the down-regulation of negative regulators of Notch signaling such as Numb (8) and Manic Fringe (MnFng; ref. 9) has been observed.

The initial murine transgenic experiments involving truncated alleles of Notch defined a role for this pathway in epithelial tumor initiation (10). More recently, activated Notch signaling has been shown to play a critical role in maintaining established murine colon (11) and ERBB2$^+$ breast cancers (12). In cell lines, Notch activation has also been implicated in promoting invasion and epithelial to mesenchymal transition (13, 14). Enhanced expression of Notch ligands has been associated with poor prognosis of breast cancers and increased metastasis in prostate cancers (15, 16). However, the molecular mechanisms of Notch signaling in promoting Notch mediated tumor progression and metastasis of primary human cancers are poorly understood.

Studies examining gene expression and mutations in primary cancers and their metastases suggest that metastatic progression occurs as primary tumors grow, develop mutations, and respond to the environment (17). Recent work suggests that metastasis may be driven by a subpopulation of cells in primary tumors (18). In addition to the linkage with ERBB2$^+$ breast cancers (12), Notch signaling is also implicated in maintaining tumorigenic subsets in colon (19) and brain

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cancers (20). In this study, we examine the role of Notch signaling in established primary human cervical cancers and define the identity and properties of cells, which promote tumor progression. These cancers, driven and sustained by HPV oncogenes (21), constitute a major malignancy among women in the developing world.

Subsets which promote epithelial cancer progression have been identified using cell-surface markers implicated in adhesion and metastasis, e.g., CD44 (22) and CD26 (23). We investigated the role of Notch in the progression of cervical squamous cell carcinomas by examining its expression and function in the context of $CD66^+$ cells. CD66 expression has been earlier associated with TGFβ and Src mediated invasion and metastasis of epithelial cancer cell lines (24–28).

The expression of CD66 has been examined in primary adenocarcinomas (29) and its function has been explored using established cell lines. In order to directly examine the functional properties of $CD66^+$ cells, we fractionate them from primary cervical cancers. In addition, we use a metastatic-lesion derived cell line to explore mechanistic features related to Notch signaling. We report that the $CD66^+$ cells, which are strikingly more tumorigenic than the $CD66^-$ cells in a range of assays, show enhanced expression of nuclear Notch targets and stemness-associated genes. Finally, using a combination of chemical inhibitors and genetic approaches, we show that the $CD66^+$ cells are markedly dependent on active Notch signaling.

Materials and Methods

Immunohistochemistry

Immunohistochemistry and immunofluorescence were carried out on formalin-fixed, paraffin-embedded tissue sections, or cryosections as described earlier (7). Primary antibodies used were α-human CD66 (BD Biosciences), Notch1 (Santa Cruz Biotechnology, sc-6014), Hes1 (Imgenex), CD45 (Abcam), and Cleaved Notch Val1744 (Cell Signaling Technology). Signal from the Cleaved Notch antibody was amplified using the TSA Signal Amplification Kit (Molecular Probes). Images were taken on ZEISS LSM 510 Meta-confocal microscope.

Flow cytometry/magnetic cell sorting

Primary human cervical squamous cell carcinoma (SCC) biopsy samples were obtained from Kidwai Hospital with appropriate institutional Ethics Committee approvals and procedures. The samples were collected and stored in Dulbecco's modified eagle's medium (DMEM; Invitrogen) with 10% FBS (Invitrogen) for not more than 2 hours at 4°C. The tissue was digested with Collagenase type IV (1 mg/ml, Sigma) to get a single cell suspension. CD66 expression was analyzed either by flow cytometry (FACS) or by magnetic sorting after Lineage depletion (α-lineage cocktail, Miltenyi Biotec) using the MiniMACS (Miltenyi Biotec) system as per the manufacturer's protocol. CaSki spheroids or monolayer cells were trypsinized to single cell suspensions and allowed to recover external-antigen expression for 2 hours. Cells were analyzed for CD66 expression on BD FACS*Calibur* or sorted on either BD FACS*Aria* or FACS*Vantage*.

Xenografts

10,000 $CD66^+$, $CD66^-$, and unsorted cells isolated from primary SCC samples or CaSki spheroids were subcutaneously injected in the flanks of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (The Jackson Laboratory) with matrigel (BD Biosciences). Tumors were collected after 6 to 8 weeks.

Cervical cancer implant model

The implant model for cancer metastasis was developed by modifying a reported procedure (30). Xenograft tumors of a primary cancer were excised under sterile conditions and stored in DMEM with 10% FBS for not more than 3 hours at 4°C. Female NOD/SCID mice were anaesthetized and the uterus exposed by an abdominal midline incision. Another small incision was made in the uterus at the level of the cervix, and a 2-mm^3 tumor fragment was sutured in place. Tumor growth and metastases were assessed after 8 weeks.

Microarray

Gene expression analysis by microarray was done on RNA extracted from sorted Lin^- $CD66^+$ or $CD66^+$ cells from primary human cervical cancers on a custom-designed 15K Array (3252 genes). The microarray-based gene expression analysis of CaSki spheroid and monolayer cells was carried out on Agilent Whole Genome Human 4 × 44 K Array in biological duplicates. The data was analyzed using GeneSpring GX (Agilent Technologies) and the statistical analysis was done with Biointerpreter (Genotypic Inc.). The microarray data reported in this article have been deposited in the Gene Expression Omnibus (GEO) database (accession no. GSE26419).

Cell culture and reagents

CaSki cell line (ATCC) was cultured in 1X DMEM containing 10% FBS and penicillin/streptomycin (100 μg/ml). Single cell suspensions of CaSki monolayer cells (0.5×10^6 cells/ml) grown in DMEM (Invitrogen) with 10% FBS (Invitrogen) were plated on polyHEMA (Sigma) coated dishes in spheroid medium, OptiMEM-Glutamax (Invitrogen) with 20 ng/ml EGF and 10 ng/ml bFGF (Peprotech). Medium was changed every third day. Inhibitors used included γ-secretase inhibitor N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT; 20 μmol/L, Sigma-Aldrich), PI3K inhibitor LY294002 (20 μmol/L, Calbiochem), and Cisplatin (2 μg/ml, Cipla). Treatments were started on the fourth day after spheroid formation, and continued for 72 to 96 hours.

Transfections

CaSki cells transfected (31) with the indicated plasmid were seeded for spheroid culture 48-hours posttransfection. All subsequent assays were done with 4-day spheroids. Plasmid constructs used included: Manic Fringe (9), dominant negative CBF1 (gift from J. Aster), dominant negative Myc or Mad/Myc (gift from R. Bernards) (31), dominant negative K179M-AKT (DN-AKT), and constitutively active Δ(4–129)-AKT or CA-AKT (gift from M. Greenberg). Stable clones of MnFng

transfected CaSki cells were made from G418-resistant colonies after MnFng expression analysis.

Immunofluorescence staining of CaSki spheroids

CaSki spheroids were grown for 7 days. The spheroids were fixed with 4% (w/v) paraformaldehyde, permeabilized and blocked with 0.1% (v/v) Triton X-100 and 5% FBS in phosphate buffered saline. The spheroids were incubated overnight with α-CD66 (BD Biosciences) followed by appropriate secondary antibody conjugates (Molecular Probes). These were imaged on ZEISS LSM 510 meta-confocal microscope.

Migration/invasion assays

These were carried out as described previously (32). Spheroid medium with 10% FBS was used as a chemoattractant.

Soft agar colony formation assay

Soft agar colony formation assay was done as described previously (3). Colonies were counted in 10 random fields at the end of 21 days (on Nikon ECLIPSE TE2000-S).

Real time PCR analysis

RNA was isolated using RNeasy Mini or Micro kits (Qiagen) and reverse transcribed by MuMLV (Invitrogen). Real-time PCR using SYBR Green (Kapa Biosystems) was done on Rotor Gene RG-3000 (Corbett Research). Relative expression was determined by normalizing to reference gene $RPLP_0$ using comparative Ct method. Primers were either designed or obtained from qPrimerDepot (NCI). The sequences are listed in the supplementary materials and methods.

Results

Metastasizing CD66$^+$ cells from primary invasive cervical cancers coexpress Notch

We sought to examine if there was a link between the expression of markers of metastasis and the Notch receptor in primary cervical cancers. An immunohistochemical staining for pan-CD66 and cleaved Notch1 proteins in primary cervical squamous cell carcinomas (SCCs) showed that these were coexpressed in the primary tumor and in migratory cells within blood vessels (Fig. 1A). Staining of serial sections showed that the CD66$^+$ cells did not express the granulocyte marker CD45 (Supplementary Fig. S1A). To ascertain if the metastasizing cells expressed CD66, we investigated the progression of a primary human cervical cancer implanted in the murine uterine cervix (Fig. 1B). Tumors in this model showed that cells which coexpressed intracellular Notch1 and CD66 were migratory (Fig. 1Ci), intravasating (Fig. 1Cii) and, metastasizing to lymph nodes (Fig. 1Ciii). Notch1 was also coexpressed with CXCR4, a well-characterized marker of metastasis (Supplementary Fig. S1B).

To further explore the role of CD66 in cervical cancer metastasis, we examined the expression of the membrane protein in primary cancers and their corresponding metastases. Typically, immunohistochemical staining for CD66 showed patches of stained cells in primary cervical SCCs, and a much greater frequency in the matched lymph-node metastases (Fig. 2A and Supplementary Fig. S2A). We noted a similarly high frequency of Notch1$^+$ cells (>90%) and Hes1$^+$ cells in most metastatic lymph nodes (Fig. 2A).

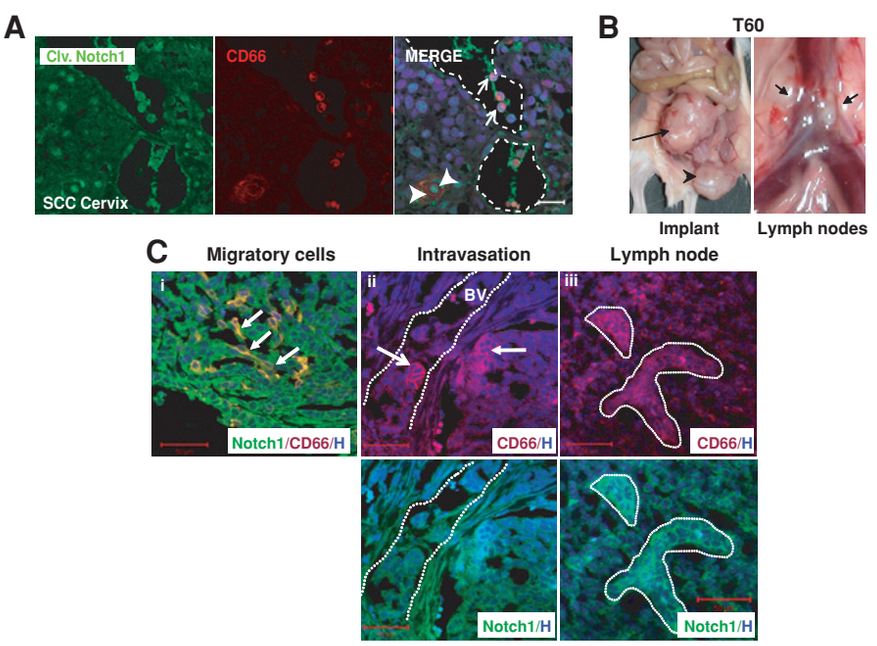

Figure 1. Metastasizing human CD66$^+$ cells express the Notch1 receptor. A, immunofluorescence staining of cleaved Notch1 (Val1744) (green), CD66 (red), and Hoechst (blue) in a cervical SCC section showing triple positive cells in the blood vessel (arrow) and in the tumor area (arrowhead). The blood vessel is outlined by a dashed line. Scale bar, 20 μm. B, xenograft tumor (8-week) from a primary cancer T60 (10^5 cells) implanted in the mouse cervix for 8 weeks (arrow) showing metastases to the body wall (arrow head) and neighboring lymph nodes (arrows). C, immunofluorescence staining of CD66 (arrows) in the T60 implant shows (i) Notch$^+$ migratory cells, (ii) cells intravasating in blood vessel (BV) marked with dotted line, and (iii) cells in lymph node sections (regions with tumor cells marked with dotted line). Scale bar, 50 μm.

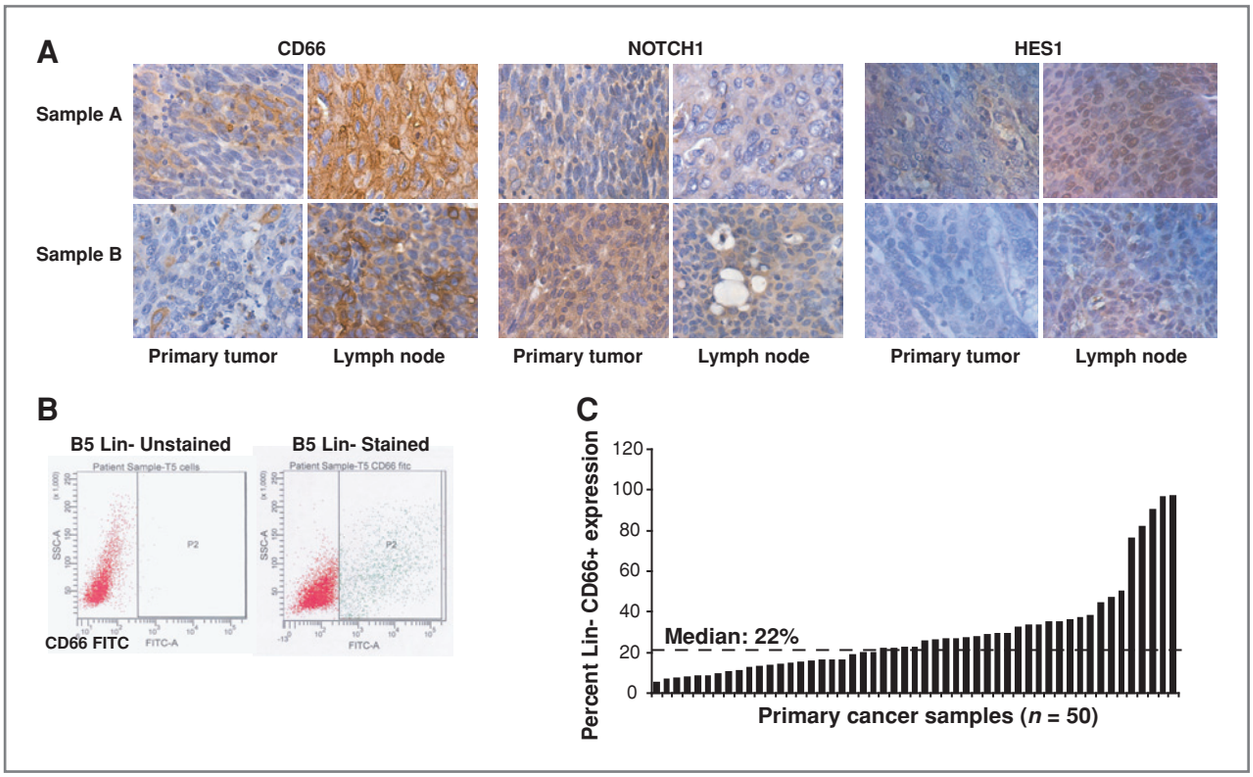

Figure 2. CD66 expression in primary human cervical cancers and their metastases. A, representative immunohistochemical staining for CD66, Notch1, and Hes1 in two primary cervical cancers and their matched lymph node metastases. B, flow cytometry analysis for CD66 expression of lineage depleted cells from a representative primary cervical tumor. C, percent Lin^- $CD66^+$ cells in 50 primary cervical cancers.

To quantify the CD66 population, we analyzed its expression in 50 primary cervical SCCs (>90% stage IIB–IV and grade II–III) by flow cytometry (Fig. 2B). To restrict our analysis to the epithelial cells, single cell suspensions of primary cancers were depleted of the $Lineage^+$ cells prior to CD66 staining. The frequency of the Lin^- $CD66^+$ population ranged from 5% to 90%, with a median frequency of around 22% (Fig. 2C).

Of the 50 primary cervical SCCs, we have follow-up records for more than six months after treatment for 15 cases. The treatment protocol of chemo- and radio-therapy is described in supplementary methods. In these 15 cases, 6 had CD66 expression below median and 9 above it. In the 6 patients with tumors that had expression of CD66 below the median level, there was no evidence of disease progression after treatment. However, 4 of the 9 cases with CD66 expression higher than the median showed residual disease after completion of treatment.

These results led us to examine fractionated cells from primary cancers for features of invasion and tumor progression.

The $CD66^+$ cells from primary invasive cervical cancers show high notch signaling and tumorigenicity

To ascertain the pathophysiologic relevance of the CD66 fraction in primary cancers, we fractionated these cells and assessed them for properties associated with malignant or metastatic cells. These included *in vitro* migration, invasion, soft agar colony formation, and *in vivo* tumor formation. We noted that the Lin^- $CD66^+$ cells were two times more migratory and invasive than the $CD66^-$ fraction (Fig. 3A). In soft agar assays, the number of colonies formed by Lin^- $CD66^+$ cells from primary cancers were three times the $CD66^-$ (Fig. 3B), showing their ability to grow and expand clonally in the absence of anchorage.

The Lin^- $CD66^+$ cells sorted from three primary cervical tumors also initiated xenograft tumors in NOD/SCID mice in four out of six cases, whereas none of the six mice injected with $CD66^-$ cells grew tumors (representative tumor in Fig. 3C). The histology of these tumors was similar to the parent primary human cervical cancer (Fig. 3C). The $CD66^+$ cells isolated from one of the murine tumors formed secondary and tertiary tumors on injection of as few as 600 cells, suggesting that these cells have the ability to sustain serial-transplantation (Supplementary Fig. S2B).

Because the Notch receptor and CD66 were coexpressed in metastasizing cells (Fig. 1), we examined the components of this pathway in sorted $CD66^+$ and $CD66^-$ cells by real-time PCR. This showed high expression of both Notch1 and its downstream effectors like *HES1*, *HEY1*, and *MYC* in Lin^- $CD66^+$ cells (Fig. 3D). Immunoblot analysis also showed increased expression of Notch1 and cMyc proteins in the

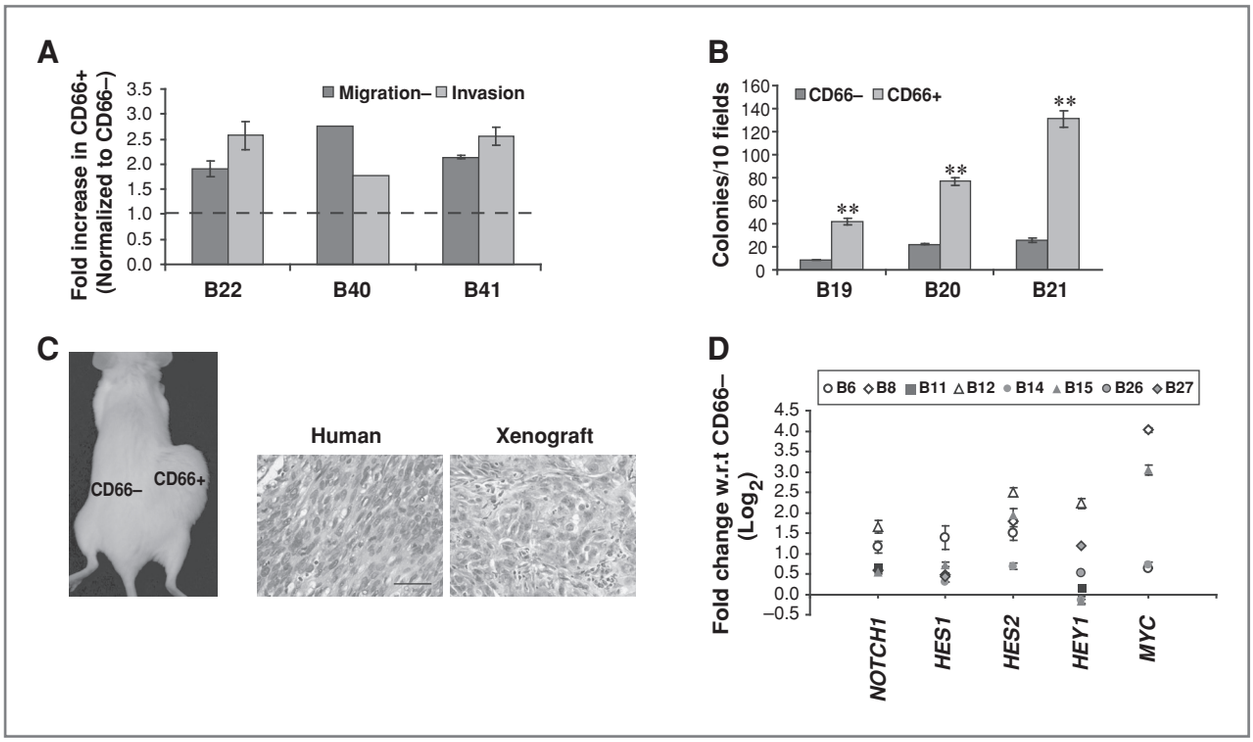

Figure 3. Fractionated CD66$^+$ subset from primary cervical carcinomas is tumorigenic and has high Notch pathway expression. A, fold increase in invasion and migration by Lin$^-$ CD66$^+$ cells as compared with Lin$^-$ CD66$^-$ cells from three primary cervical cancers (error bars show std. dev., $n = 3$). B, fold increase in the number of soft agar colonies formed by Lin$^-$ CD66$^+$ cells as compared with Lin$^-$ CD66$^-$ cells from primary tumors (error bars show std. dev., $n = 3$). C, the mouse shows a representative tumor formed by 10^4 CD66$^+$ cells from a primary tumor B21. H&E staining shows the morphology of the B21 human tumor and the xenograft grown in NOD/SCID mouse. Similar tumors were seen with four of six CD66$^+$ injections from three primary cancers, whereas none of the six CD66$^-$ injections formed tumors. Scale bar, 50 μm. D, real-time PCR analysis for the indicated notch pathway genes in Lin$^-$ CD66$^+$ cells sorted from 8 primary cancers. The values are normalized to the expression in Lin$^-$ CD66$^-$ cells. *, $P < 0.01$ and **, $P < 0.001$ (Student's t-test).

CD66$^+$ population. However, levels of the cytoplasmic Notch effector protein pAKT changed only marginally (Supplementary Fig S2C). These results indicate that fractionated CD66$^+$ cells from primary cancers are more invasive and tumorigenic than the CD66$^-$ population; and, have enhanced Notch signaling.

Gene expression signature of CD66$^+$ cells from primary cancers

Gene expression signatures of metastasis have typically focused on transcripts regulating invasion, growth factor response, angiogenesis, etc. (17). More recently, metastasis has also been linked to stemness (33). Because pathways such as Notch and Wnt signaling are known to regulate stem cell function and tumorigenesis (34), we designed an appropriate microarray to explore their expression in the CD66$^+$ population from primary cancers. The microarray contained genes involved in Notch and Wnt signaling and many of their reported interactors. The microarray-chip also had genes involved in stemness and epithelial differentiation and appropriate negative and positive controls.

In accordance with the enhanced migration and invasion by CD66$^+$ cells (Fig. 3A), sorted Lin$^-$ CD66$^+$ (PS-M1+) from a primary cervical SCC sample PS-M1 showed increased expression of genes involved in invasion such as matrix metalloproteinases (MMP) as compared to the Lin$^-$ CD66$^-$ (PS-M1-) cells (Fig. 4 and Supplementary Fig. S3A). Consistent with this, CD66 is reported to induce MMP expression in pancreatic cancer cell lines (35).

The microarray analysis showed that genes whose expression increased by more than two-fold included canonical Notch and Wnt targets (Fig. 4). We also noted an increase in expression of genes associated with stemness (*NANOG*, *OCT4*, etc.) and drug transport (*ABCC3* etc.). A comparison of PS-M1- with the microarray profile of Lin$^-$ CD66$^+$ cells from 3 other SCC samples (PS-M2+, M3+ and M4+) revealed a similar trend with an average of two to three-fold increase in metastasis related transcripts, Notch targets, and stemness-associated genes (Supplementary Fig. S3B, C).

Poorly differentiated tumors with basal-cell like characteristics are believed to be more malignant and aggressive. We thus sought to examine if the CD66$^+$ cells expressed genes associated with undifferentiated or basal epithelial cells. An analysis of the cytokeratin expression profile showed that the CD66 population expressed high levels of CK19 and reduced levels CK10 (Supplementary Fig. S3D). CK19 and CK10 have been shown to be cervical epithelium basal and differentiated cell markers, respectively (9). The microarray results were validated by RT-PCR using independent SCC samples

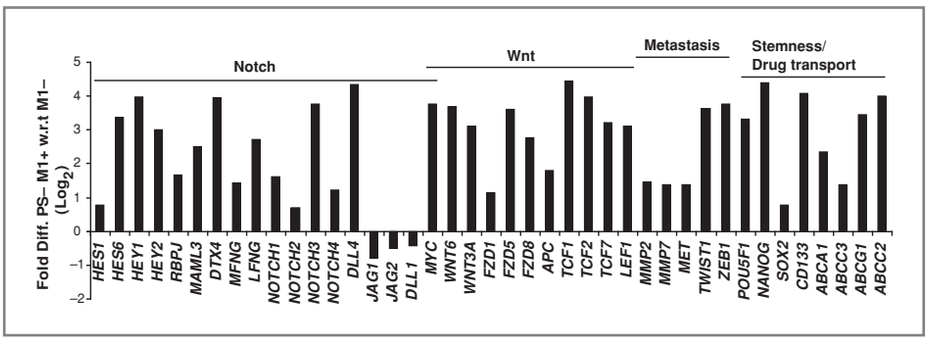

Figure 4. Microarray analysis of sorted Lin$^-$ CD66$^+$ cells from a primary cancer shows features of Notch pathway, metastasis, and stemness. The graph shows fold difference (Log_2) in expression of some genes that change by two-fold or more in PS-M1+ sample as compared with PS-M1-. χ^2 $P < 0.002$ (for notch pathway).

(Supplementary Fig. S3E) and they also correlated with the Notch pathway RT-PCR described earlier (Fig. 3D).

Our microarray data indicated that CD66$^+$ cells have a gene signature consistent with high Notch activation and features of stemness. However, it has been noted, for example, that hematopoietic stem cells show high Notch and Notch reporter activity (36), but are unaffected by a loss of Notch signaling (37). We thus examined if the CD66$^+$ cells with high Notch expression are dependent on active Notch signaling.

CD66$^+$ cells from primary cancers depend on active Notch signaling

We measured the effect of Notch inhibition on the proliferation of CD66$^+$ cells isolated from primary cancers and on the viability and expression of CD66 in these cancers. γ-secretase inhibitors (GSI) have been used as chemical tools to inhibit Notch pathway function even though they may have broader targets (38). We therefore examined the effect of the GSI DAPT on clonal proliferation of the CD66$^+$ cells in soft agar assays.

The addition of DAPT led to a marked reduction in the colony forming ability of Lin$^-$ CD66$^+$ cells (Fig. 5A), indicating that Notch signaling is required for the growth of CD66$^+$ cells.

Culturing primary cells with DAPT did not affect their viability (Fig. 5B), but markedly reduced the CD66$^+$ fraction (Fig. 5C). Inhibition of Notch signaling has earlier been shown to sensitize cervical cancer cell lines to cisplatin induced-apoptosis (39). Our monitoring of initial clinical responses suggested that high expression of CD66 is linked to poor response to cisplatin and radiation. We thus assessed the effect of cisplatin and DAPT *in vitro*. We noted that cisplatin affected the overall viability, but had no effect on CD66 expression (Figs. 5B, C). However, the combination of two drugs showed both a marked loss in viability and CD66 expression (Figs. 5B, C). The CD66$^+$ fraction thus becomes susceptible to cisplatin in the presence of Notch inhibitors. The results in this figure show that CD66$^+$ cells from primary cancers show a marked dependence on active Notch signaling.

CD66$^+$ cells from CaSki spheroids are dependent on canonical Notch signaling

Recently, established cell lines have also been reported to contain subsets with distinctive tumor propagating properties (18). The signals that regulate the emergence and maintenance of these populations are poorly understood. We used a metastatic lesion derived cervical-cancer cell line CaSki (40) to explore the potential emergence of CD66$^+$ cells and the downstream effectors of Notch in regulating CD66 expression.

Typically, standard monolayer cultures of CaSki did not show detectable CD66 protein or transcripts. However, cells grown as spheroids in a defined medium without serum showed marked CD66 expression in 4% to 6% of cells (Figs. 6A, B). To examine whether in the context of CD66 expression there is a similar upregulation of genes associated with Notch signaling and tumor progression, we undertook a microarray-based analysis of CaSki monolayer and spheroids (Supplementary Fig. S4). We noted that the spheroid cultures had high activation of Notch pathway and of metastasis-associated genes (Fig. 6C). RT-PCR based analysis also showed increased expression of Notch target genes in sorted CD66$^+$ cells from the spheroids (Fig. 6D). Further, western blotting analysis indicated that the CD66$^+$ cells have high Notch and cMyc levels, but low amounts of pAKT (Fig. 6E).

We next examined if the CaSki-derived CD66$^+$ cells were functionally similar to their counterparts from primary cancers (Fig. 3). We noted that the CD66$^+$ cells isolated from CaSki spheroids showed enhanced tumor formation *in vivo* (Fig. 7A), increased invasive ability (Fig. 7B) and enhanced ability to proliferate clonally in soft agar assays *in vitro* (Fig. 7C).

In order to examine whether the Notch pathway plays a role in maintaining the CD66$^+$ cells, and the components of the pathway involved in this process, we used various approaches to inhibit this pathway. This included using DAPT; blocking Jagged-mediated Notch activation by over-expressing Manic Fringe (9); inhibiting nuclear Notch signaling with dominant negative CBF1; and, lastly by inhibiting the downstream CBF1-target cMyc (31, 41). Because earlier work on Notch in cervical cancers has primarily focused on PI3K/AKT signaling (42), the effect of inhibiting this pathway was also examined.

CaSki cells expressing Manic Fringe (MnFng), showed a two-fold reduction in CD66 expression in FACS analysis. A similar reduction was seen in cells cultured with DAPT (Fig. 7D). The effect of DAPT on Notch inhibition was confirmed by immunoblotting-treated spheroids for cleaved Notch (Supplementary Fig. S5A). Similar to the primary cancers, CD66 expression in CaSki spheroids was also insensitive to cisplatin (Supplementary Fig. S5B). Further, MnFng expressing CaSki cells generated very small tumors that were unable

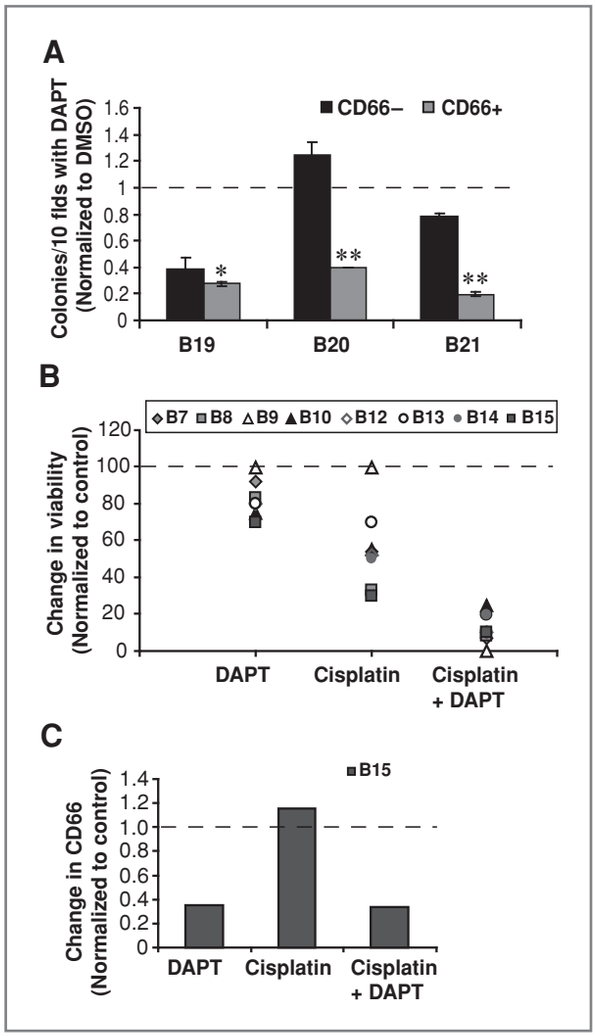

Figure 5. Sorted CD66$^+$ cells from primary human cancers depend on active Notch signaling. A, fold change in the number of soft agar colonies formed by Lin$^-$ CD66$^+$ and CD66$^-$ cells from three primary cancer samples ($n = 3$ each) on treatment with DAPT for the last 7 days of culture as compared with dimethyl sulfoxide (DMSO) treated control cells. *, $P < 0.01$ and **, $P < 0.001$ (Student's t-test). B, percent change in viability (trypan blue exclusion assay) of primary cancer cells on treatment with DAPT, Cisplatin, or a combination of both for 24 hours as compared with DMSO treated control cells. C, fold change in CD66 expression in a representative primary cervical cancer when treated as described above.

to implant and grow in the mouse cervix (Supplementary Fig. S5C). The small MnFng tumors mostly had necrotic cells (Supplementary Fig. S5C) and showed a three-fold lower fraction of CD66$^+$ cells (Fig. 7E).

DN-CBF1 transfected CaSki spheroids showed a decline in the transcription of targets like *HES1* and *MYC*, though no change was seen in the unrelated *ABCC3* (Supplementary Fig. S5D). There was a marked reduction in both CD66 protein (Fig. 7F) and *CD66c* mRNA levels (Supplementary Fig. S5E). A similar reduction in CD66 expression was noted in cells transfected with Mad/Myc (Fig. 7H and Supplementary Fig. S5E), a strong suppressor of Myc-induced transcription (31). We noted that CaSki CD66$^+$ cells expressed high amounts of cleaved Notch and Myc but the pAKT levels were reduced (Fig. 6E). Moreover, inhibiting PI3K signaling with either a chemical (Fig. 7F) or genetic inhibitor, or activating it with constitutively active AKT had no effect on CD66 expression (Supplementary Fig. S5F).

Discussion

The key observation in this study is the linking of Notch pathway activation and function to CD66, a molecule previously implicated in tumor progression and metastasis of cell lines (24, 35, 43). Our results show that CD66 is expressed in primary cervical cancers and its expression is enhanced in metastases (Fig. 2A). We also note that cells which are invading, intravasating, and accumulating at metastatic sites such as lymph nodes show marked CD66 expression (Fig. 1C). Moreover, these CD66$^+$ cells coexpress the Notch1 receptor (Fig. 1A, C). Because there has been no direct functional analysis of CD66$^+$ cells from clinical lesions, we examined the properties and status of Notch signaling in cells fractionated from primary human cancers. The CD66$^+$ cells from primary cervical cancers show features of invasion, clonal proliferation, and tumor initiation in immunodeficient mice (Fig. 3). These data are consistent with an involvement of CD66$^+$ cells in promoting tumor progression and metastasis.

Cells which are dependent on Notch signaling in both murine models and human malignancies such as colon cancers (19) and gliomas (20) have recently been linked to properties associated with stem and stem-like cells. In Figure 4 we show that CD66$^+$ cells from primary human cancers not only have features of canonical Notch signaling but also show enhanced expression of genes associated with stemness and metastasis. Our microarray showing enhanced features of Notch and Wnt signaling pathways (Fig. 4) broadens the association of oncogenicity and self-renewal in the context of Notch-mediated cancers. This association with self-renewal is supported by our data showing serial transplantation of xenografts by CD66$^+$ cells (Supplementary Fig. S2B).

We have previously reported a prosurvival function of Notch signaling in human cervical cancers (3, 42). A crosstalk between Notch signaling and PI3K/AKT has also been seen in cervical cancer cell lines (13, 32, 44). Here, we broaden the scope of Notch signaling by showing that cells that undergo clonal proliferation are characterized by activated Notch signaling and are dependent on this pathway (Figs. 4 and 5A). The pattern of staining in metastases suggests that such cells are preferentially selected during cancer progression *in vivo* (Figs. 1, 2). The data in this study also links canonical Notch signaling to the regulation of properties of CD66$^+$ cells (Figs. 4 and 7F). Recent work suggests that metastatic cells in murine colon cancers also rely on canonical Notch signaling (45). Murine breast cancers that respond to Notch inhibitor *in vivo* are known to have high CBF1 expression (46).

Currently, considerable attention is being focused on the existence, identity and properties of cancer stem cells. There

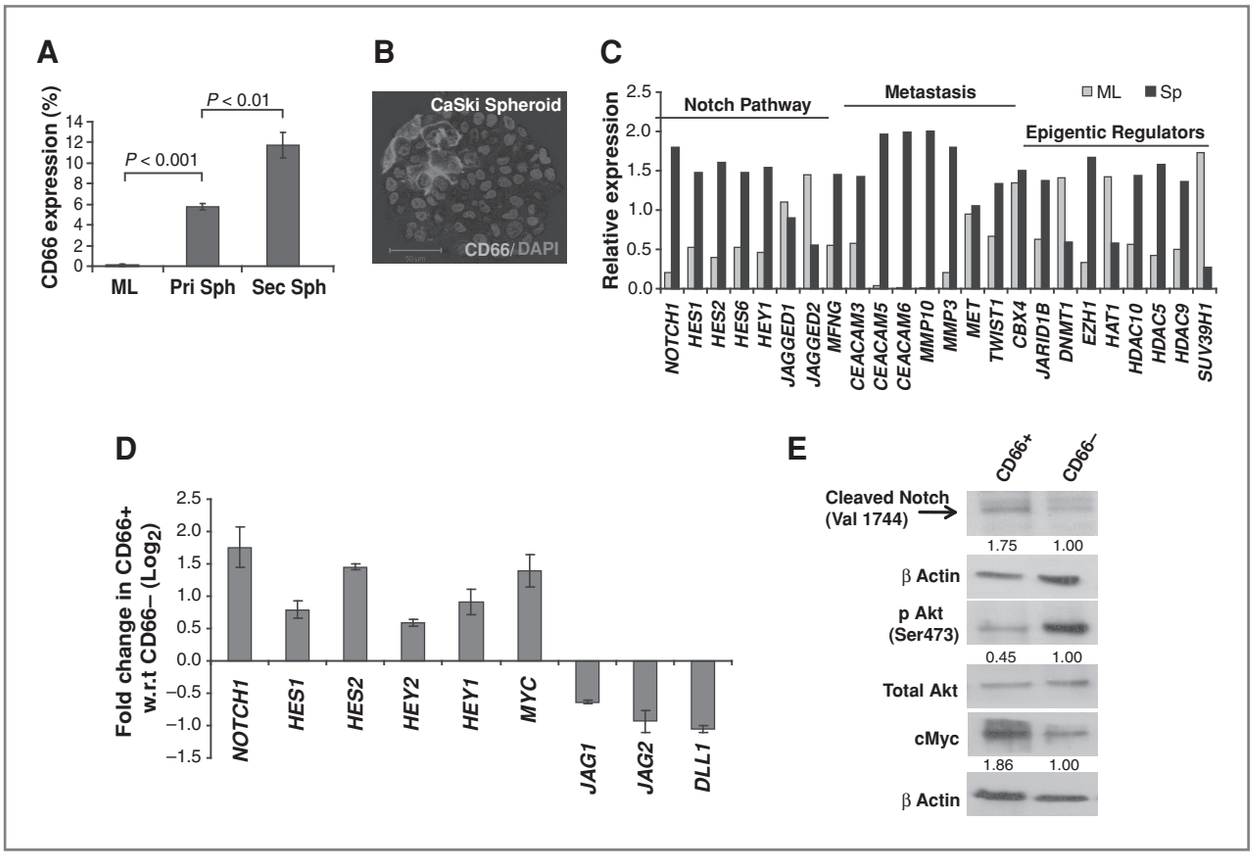

Figure 6. CD66$^+$ cells enriched in CaSki spheroids show enhanced nuclear Notch signaling. A, flow cytometry analysis for CD66 expression in CaSki spheroids and monolayer cultures (error bars show std. dev., $n = 4$; P-value from Student's t-test). B, representative immunofluorescence staining for CD66 (green) and DAPI (blue) in a CaSki spheroid. C, microarray-based gene expression analysis of monolayer (ML) and spheroid (Sp) cells showing change in expression of Notch pathway genes, metastasis-related genes, and epigenetic regulators. D, fold change in the expression of indicated Notch pathway genes in CaSki CD66$^+$ cells by RT-PCR. The values are normalized to the expression in CD66$^-$ cells. For all genes $P < 0.05$ (Student's t-test). E, immunoblot analysis for expression of the indicated proteins in lysates from CD66$^+$ and CD66$^-$ cells.

also seems to be a stage-specific heterogeneity in the markers expressed by putative cancer stem cells (23). Because a recent study has indicated that cervical cancer stem cells are CD44$^+$ (47), we suggest that a detailed exploration of CD44, CD66, CD133 and other possible markers be undertaken prior to any definitive assignment of features and properties of cervical-cancer stem cells.

Chemical γ-secretase inhibitors (GSI) of Notch signaling have been shown to reduce the putative cancer stem cell populations in glioma and colon cancer sphere cultures (19). In order to examine this question in a more direct manner, we fractionated CD66$^+$ cells from primary human cancers and tested the effect of GSI on these cells. We note that a GSI inhibits the proliferation of CD66$^+$ cells in assays that measure clonal growth in soft agars (Fig. 5A). GSIs have also been reported to synergize with cisplatin, a known chemotherapeutic drug, in preventing the proliferation of established cancer cell lines (39). Our data shows that GSIs reduce the expression of CD66 in primary cancers and synergize with cisplatin in affecting the total cell viability (Fig. 5B, C). The *ex-vivo* susceptibility of CD66$^+$ cells from primary cancers to GSI and the possible link of CD66 expression in determining therapeutic response lays the foundation for a well-designed large scale clinical evaluation of GSIs.

Whereas numerous studies have reported features of deregulated Notch expression and signaling during cervical cancer progression (5–7, 31, 32, 42), some experiments with human cervical cancer cell lines have suggested a tumor suppressive role for Notch signaling (48, 49). The results of this study strengthen the prooncogenic role of Notch signaling in primary human cervical cancers by defining a cellular subset that is markedly tumorigenic and dependent on this pathway. Our microarray results (Fig. 4) reveal a high expression of DLL4 in the CD66$^+$ cells. An estimation of the DSL ligand protein levels would be required to draw a more definitive conclusion on the process of Notch activation in these cells. Because an oncogenic function of Notch signaling has been shown in diverse human cancers, the results in this study have widespread significance in the design of novel therapies.

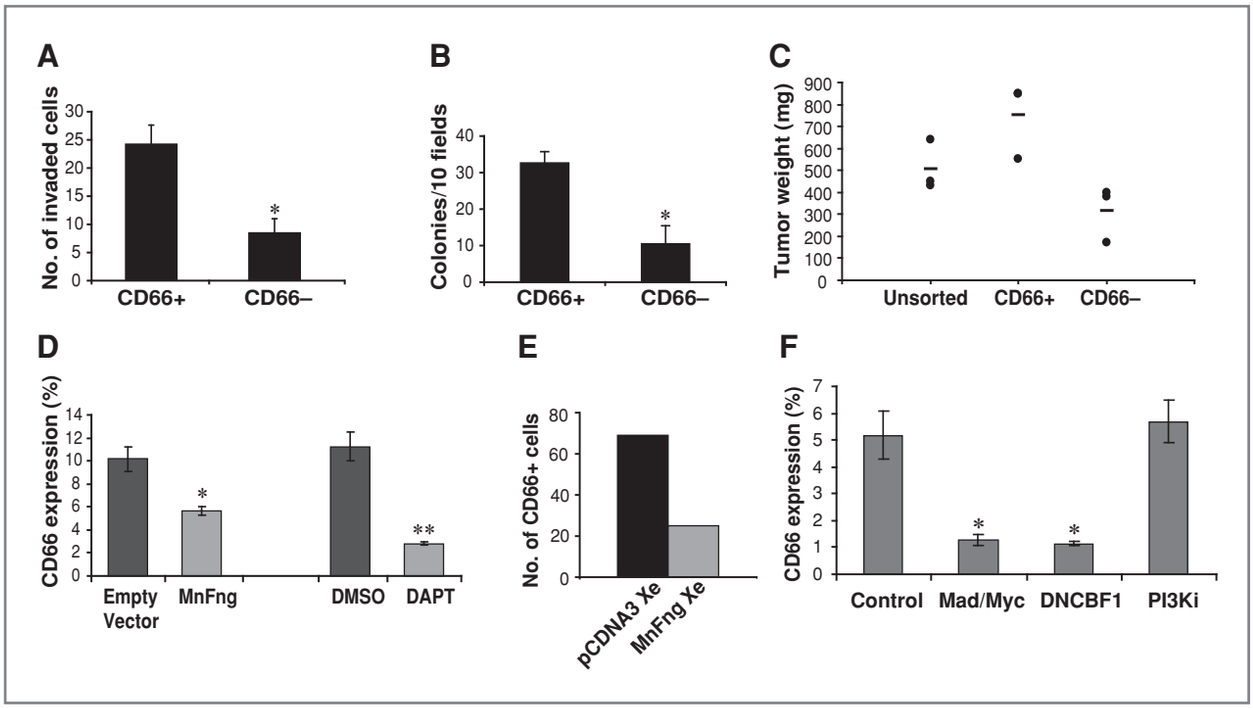

Figure 7. Tumor-promoting CD66$^+$ cells from CaSki are maintained by canonical Notch signaling. A, fold increase in invasion and migration by CD66$^+$ cells as compared with CD66$^-$ cells (error bars show std. dev., $n = 3$). B, fold increase in the number of soft agar colonies formed by CD66$^+$ cells as compared with CD66$^-$ cells (error bars show std. dev., $n = 3$). C, average weight of 6-week tumors formed by 10^4 CD66$^+$, CD66$^-$, and unsorted CaSki spheroid cells in NOD/SCID mice ($n = 3$ plotted). D, percent CD66 expression by FACS in spheroids of CaSki cells stably expressing pcDNA3 empty vector (Neo) or Manic Fringe (MnFng) and in DAPT treated CaSki spheroids. E, total number of CD66$^+$ cells in 10 low power fields of pCDNA3 and MnFng xenografts. F, flow cytometry of CD66 expression in CaSki spheroids transiently transfected with DN-CBF, Mad/Myc, and empty vector ($n = 3$); or, on treatment with LY294002. The average of two separate transfections is plotted (error bars represent SEM). For the chemical inhibitor, $n = 5$. *, $P < 0.05$, **, $P < 0.001$ (Student's t-test).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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