Regulation of the Embryonic Morphogen Nodal by Notch4 Facilitates Manifestation of the Aggressive Melanoma Phenotype

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Abstract

Metastatic melanoma is an aggressive skin cancer associated with poor prognosis. The reactivation of the embryonic morphogen Nodal in metastatic melanoma has previously been shown to regulate the aggressive behavior of these tumor cells. During the establishment of left-right asymmetry in early vertebrate development, Nodal expression is specifically regulated by a Notch signaling pathway. We hypothesize that a similar relationship between Notch and Nodal may be reestablished in melanoma. In this study, we investigate whether cross talk between the Notch and Nodal pathways can explain the reactivation of Nodal in aggressive metastatic melanoma cells. We show a molecular link between Notch and Nodal signaling in the aggressive melanoma cell line MV3 via the activity of an RBPJ-dependent Nodal enhancer element. We show a precise correlation between Notch4 and Nodal expression in multiple aggressive cell lines but not poorly aggressive cell lines. Surprisingly, Notch4 is specifically required for expression of Nodal in aggressive cells and plays a vital role both in the balance of cell growth and in the regulation of the aggressive phenotype. In addition, Notch4 function in vasculogenic mimicry and anchorage-independent growth in vitro is due in part to Notch4 regulation of Nodal. This study identifies an important role for cross talk between Notch4 and Nodal in metastatic melanoma, placing Notch4 upstream of Nodal, and offers a potential molecular target for melanoma therapy.

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Introduction

Metastatic melanoma is a highly aggressive skin cancer associated with poor clinical outcome. One key feature is the expression of a plastic, multipotent cellular phenotype resembling embryonic stem cells in its molecular profile (1). Both stem cells and aggressive melanoma cells participate in bidirectional communication with the microenvironment, which can profoundly influence cell behavior (2, 3). Cancer cells can exploit normally dormant embryonic pathways to promote tumorigenicity. Understanding these embryonic signals and the regulatory programs that reactivate them holds significant potential for cancer therapies, including those for melanoma.

Notch and Nodal are 2 signaling pathways that participate in embryonic stem cell maintenance and whose expression and/or activation correlates with melanoma progression (3–5). Nodal is a TGFβ superfamily member that typically associates with type I (ALK4/5/7) and type II (ActRIIB) activin-like kinase receptors to activate signaling through Smad2/3/4 and promote a gene transcriptional program that includes Nodal itself and the Nodal antagonist Lefty (6). Nodal signaling regulates multiple embryonic processes, but Notch is not typically expressed in normal adult tissues including melanocytes (2). Reexpression of Nodal has been observed in melanoma as well as breast and endometrial carcinomas (2, 3, 7, 8). Nodal signaling plays an important role in the aggressive nature of metastatic melanoma, as pathway inhibition blocks tumorigenic capacity and plasticity of aggressive human melanoma cells (2, 3).

The Notch receptor family consists of 4 single transmembrane receptors (Notch1–4) and 5 membrane-bound ligands (Delta-like1, 3–4 and Jagged1–2). Structurally the 4 Notch receptors are somewhat similar. Notch1 and Notch2 have high homology, whereas Notch3 and Notch4 retain some structural differences. Compared with Notch1, Notch4 has a shortened extracellular domain and lacks the intracellular transactivation domain (TAD) and cytokine response sequence (NCR) (9, 10). For all the Notch receptors, signaling requires contact between 2 cells and is propagated directly by the Notch receptor intracellular domain (ICD) that is released into the cytoplasm by sequential proteolytic cleavage steps.
Notch and Nodal in Melanoma

(10, 11). The active ICD can bind coactivator proteins, such as recombination signaling binding protein-J (RBPJ) and mastermind-like proteins, and form a nuclear transcriptional activator complex. Notch signaling regulates key aspects of embryogenesis, as well as adult tissue homeostasis including maintenance of the melanocyte precursor population (12, 13). Aberrant Notch signaling can promote or inhibit oncogenesis depending upon the Notch receptor profile and the tumor or cell type (14). Notch targets implicated in cancer include c-myc, cyclin-D1, and p21/Waf1 (15).

A direct connection between the Nodal and Notch signaling pathways occurs in early embryogenesis during vertebrate body plan formation. Early in mouse development, Nodal is expressed around the transient embryonic structure, termed the Node (a group of cells that secrete inductive cues to facilitate body plan organization), and is required for gene expression that regulates left-right asymmetry (16). Notch pathway components are similarly expressed (17, 18), and Notch signaling is necessary for Nodal expression at this developmental stage (19, 20). This relationship is direct, as the Notch–ICD complex can bind and drive an RBPJ-dependent enhancer element (termed the Node-specific enhancer NDE) upstream of the Nodal gene (19, 20). The noncoding NDE region is highly conserved, indicating the possibility that a relationship also exists between Notch and Nodal in humans. Although the embryonic Node itself has no direct connection to cancer biology, we hypothesized that the relationship between Notch and Nodal may be conserved or reestablished in melanoma, in which Nodal is reactivated (2, 3, 8). Preliminary evidence suggests that cross talk exists between Notch4 and Nodal in an aggressive melanoma cell line (21, 22). In the present study, we explore the precise relationship between Notch4 and Nodal expression and how this cross talk influences aggressive melanoma cell behavior.

Materials and Methods

Cells
Melanoma cell lines UACC1273, c81-61, and C8161 were obtained from the University of Arizona (1987/1999). MV3 cells were a gift from Dr. van Muijen (University Hospital Nijmegen; 2006). WM852 cells were a gift of Dr. Herlyn (The Wistar Institute; 2001). SK-MEL-28 cells were from American Type Culture Collection (ATCC; 2010). Cell lines were authenticated by short tandem repeat genotyping by PCR amplification.

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Constructs/luciferase assays
pGL3-basic was purchased from Promega. pGL3p containing mouse Nodal NDE was a gift from Dr. Bruneau (UCSF; ref. 23). RBPJ binding sites were mutated using the QuikChange Site-Directed Mutagenesis kit (Stratagene). Plasmids were transfected into MV3 and C8161 cells, using Arrest-In reagent (Thermo Scientific Open Biosystems). Luciferase assays were carried out after 48 hours (Promega). Each parameter was assayed in quadruplicate, and experiments were done 3 times.

Reverse transcription and PCR
Total RNA was isolated from cells, using the PerfectPure RNA Cell Isolation kit (5Prime). Reverse transcription of total RNA, semiquantitative PCR (parameters in Supplementary Table 1), and real-time PCR were done as previously described (2, 3). TaqMan gene expression primer/probe sets (Applied Biosystems) utilized were Nodal (Hs00250630_s1), Notch1 (Hs00131877_m1), Notch2 (Hs00225747_m1), Notch3 (Hs01066432_m1), Notch4 (Hs00270200_m1), and RPLPO large ribosomal protein (433761F). Samples were assayed in triplicate and experiments were done 3 times.

Antibodies/Western blot analysis
Collection and quantification of protein lysates, SDS-PAGE gel electrophoresis, and Western blot analysis was carried out as described (3). Antibodies and working dilutions are in Table 1, and real-time PCR were done as previously described (2, 3). TaqMan gene expression primer/probe sets (Applied Biosystems) utilized were Nodal (Hs00250630_s1), Notch1 (Hs00131877_m1), Notch2 (Hs00225747_m1), Notch3 (Hs01066432_m1), Notch4 (Hs00270200_m1), and RPLPO large ribosomal protein (433761F). Samples were assayed in triplicate and experiments were done 3 times.

Immunofluorescence
Cells were fixed in ice-cold methanol, blocked in 5% bovine serum albumin, and incubated in primary antibodies (see Supplementary Table 2), followed by AlexaFluor-488 and AlexaFluor-594 secondary antibodies (1:400; Invitrogen). Coverslips were mounted with Vectashield containing DAPI (Vector Laboratories). Fluorescence was visualized on a Zeiss-Meta700 confocal microscope and images were captured using Zeiss ZEN software (Carl Zeiss). Cells were counted in 7 separate fields under a 25× Zeiss LD Planapo2.5x/0.8 Imm Corr objective, and mean ± SD presented graphically.

Immunohistochemistry
Serial sections of a melanoma tissue array (ME1003) were purchased from US Biomax and stained for Notch4 or Nodal expression as previously described (3) using primary antibody for 60 minutes. Isotype IgG at the same concentration was used as controls (Jackson ImmunoResearch Laboratories). Images were captured using a Leica DM4000B microscope equipped with a DFC480 CCD camera (Leica Microsystems). Tissue staining was determined from the array datasheet and staining was scored as follows: none, weak (<25%), moderate (25%–50%), and strong (>50%). Immunostaining and scoring of results were carried out by a trained pathologist (L.S.). In total, 61 melanoma tissue samples were evaluated. Statistical significance was determined using Z-test for 2 proportions (95% confidence level).

siRNA transfection
Cells were transfected with negative control #1, Notch1 (s9635), Notch2 (s9637), Notch3 (s9641), or Notch4 (s9644)
Silencer Select Predesigned siRNAs, using siPORT-NeoFX (Applied Biosystems/Ambion). Cells were retransfected after 48 hours and analyzed immediately (RNA) or 48 hours later (protein). Experiments were done 3 times.

**Antibody treatments**

C8161, MV3, and SK-MEL-28 cells seeded at confluence were antagonized with a goat anti-human Notch4 neutralizing antibody (24); Supplementary Table 2) or goat whole molecule IgG (Jackson ImmunoResearch Laboratories) at 5 μg/mL. Antibody or IgG was added at 24-hour intervals for a total incubation of 72 hours. In some experiments, 100 ng/mL recombinant human Nodal (R&D Systems) was also added. Cells were harvested for real-time reverse transcriptase PCR, Western blotting, or flow cytometry. Preservative was removed from antibody prior to use.

**Flow cytometry**

Cells were treated with antibody as described and then harvested at 24-hour time-points for ViaCount or Nexin assays (Guava Technologies/Millipore). Gating parameters were set using untreated cells. Triplicate samples were averaged for each data point and experiments were done 3 times.

**Vasculogenic mimicry assay**

3D-collagen matrices were prepared as previously described (25). Cells were seeded onto matrices either untreated or treated with goat IgG or anti-Notch4 antibody (3 μg/mL) plus or minus recombinant human Nodal (100 ng/mL). Antibody was added daily for 96 hours, and cultures were imaged using a Zeiss inverted microscope and Hitachi HV-C20 CCD camera (Hitachi Denshi).

**Clonogenic assay**

Assays were prepared in triplicate as previously described (3), except cells were either untreated or pretreated with goat IgG or anti-Notch4 antibody (3 μg/mL for 72 hours) plus or minus recombinant human Nodal (100 ng/mL). A total of 5,000 viable cells were seeded per well and macroscopic cell clusters (>50 cells) were scored after 3 weeks. Triplicate wells were averaged and presented as a percentage (mean ± SD) of control. Experiments were done 3 times.

**Results**

**An embryonic Nodal gene enhancer element links the Notch and Nodal pathways in the aggressive melanoma cell line MV3**

In an initial effort to identify a molecular link between Notch and Nodal in melanoma, we asked whether an artificial reporter of the NDE region exhibits activity in the aggressive melanoma cell line MV3, which expresses Nodal mRNA and protein. The NDE is located 10-kb upstream of the human Nodal gene and contains 2 conserved RBPJ binding sites (Fig. 1A; refs. 19, 20). In early mouse development, binding to this enhancer region regulates Nodal gene expression in an RBPJ-dependent manner. We reasoned that this embryonic Notch–Nodal relationship may be duplicated in melanoma, in which Nodal is reexpressed (2, 3, 8).

Luciferase reporter constructs containing either wild-type NDE (WT-NDE) sequence (23) or NDE sequences with mutations in one or both RBPJ binding sites (Fig. 1B) were transfected into MV3 cells. Compared with WT-NDE, mutations in either the first or second RBPJ binding site (MT1-NDE or MT2-NDE) diminished luciferase activity to approximately half of wild-type levels.

**Figure 1.** A Nodal enhancer element drives luciferase expression in MV3 cells in an RBPJ-dependent manner. A, representation of the Nodal gene locus with upstream NDE region and the 2 putative RBPJ binding sides. B, WT-NDE, single mutant (MT1-NDE; MT2-NDE), and double mutant (DM-NDE) reporter constructs utilized. C, ability of wild-type and mutant NDE constructs to drive luciferase activity in MV3 cells. pGL3-basic (NEGATIVE) was a negative control. *, significant difference from WT-NDE (P < 0.05); **, significant difference from MT1-NDE/MT2-NDE (P < 0.05). Graph is an average (mean ± SEM) of 3 independent experiments.
(MT1-NDE, 45% ± 3%; MT2-NDE, 43% ± 1%; P < 0.05), whereas mutations in both RBP4 binding sites (DM-NDE) severely reduced luciferase activity, significantly lower than MT1-NDE or MT2-NDE levels (15% ± 1%; P < 0.05) (Fig. 1C). The same trend was also observed in C8161 cells (data not shown). These findings collectively suggest the possibility that the NDE region may contribute to driving Nodal reexpression in these aggressive cells via a Notch–RBP4 pathway. This initial connection between Nodal and Notch in MV3 cells provided the platform for our in-depth investigation of Notch–Nodal cross talk in multiple melanoma cell lines.

**Notch4 expression correlates with Nodal expression in multiple aggressive melanoma cell lines**

A relationship between Nodal expression and melanoma aggressiveness has been established, and in vitro Nodal expression is predominant in aggressive melanoma cells compared with poorly aggressive counterparts (3). Certain Notch receptors have been evaluated in melanoma compared with melanocytes (5, 26). To assess the complete Notch receptor expression profile of melanoma cells, we surveyed mRNA and protein levels in 4 aggressive (C8161, MV3, SK-MEL-28, and WM852) and 2 poorly aggressive (UACC1273 and c81-61) cell lines (Fig. 2A and B). By semiquantitative PCR, *Notch1* and *Notch2* genes were similarly expressed in all the cell lines (Fig. 2A). *Notch3* was predominantly expressed in MV3 and WM852 cells. However, Notch4 mRNA expression was observed only in the 4 aggressive cell lines, consistent with Nodal transcripts. At the protein level, Notch1 and Notch2 were detected in all the cell lines (Fig. 2B). In contrast, Notch3 protein was observed both in poorly aggressive c81-61 cells and in aggressive MV3 and WM852 cells. Of note, Notch4 protein was detected exclusively in the 4 aggressive cell lines, in agreement with Nodal protein.

A fundamental role of Notch signaling is in regulation of cell fate and behavior during embryogenesis (11). Notch signaling can become spatially restricted to a subset of cells in a developing tissue by asymmetric cell division or lateral inhibition (27). We investigated whether Notch4 and Nodal expression is restricted to a subpopulation of aggressive melanoma cells. Confocal microscopy of C8161, MV3, and SK-MEL-28 cultures with Nodal (green) and Notch4 (red) antibodies (Fig. 2C; Supplementary Fig. 1) revealed that protein expression was indeed limited to a subpopulation of cells. We detected Nodal protein in approximately one-third of cells (C8161, 39% ± 6%; MV3, 29% ± 11%; SK-MEL-28, 29% ± 7%; Fig. 2D). Notch4 protein was detected in up to a quarter of cells (C8161, 25% ± 3%; MV3, 18% ± 8%; SK-MEL-28, 18% ± 2%). Importantly, Nodal and Notch4 coexpression was also observed in a subset of cells (C8161, 10% ± 3%; MV3, 10% ± 8%; SK-MEL-28, 11% ± 2%), suggesting that cross talk between these pathways occurs in cell subpopulations. In all cell lines, staining was predominant in the cytoplasm, with some membrane and occasional nuclear localization. Furthermore, some cells exhibited Nodal and Notch4 colocalization (yellow; arrowheads in Fig. 2C and in Supplementary Fig. 1A–C).

We also carried out immunohistochemistry on serial sections of a human melanoma tissue array to address whether Notch4 and Nodal expression was correlated with melanoma progression. Results indicated that strong immunostaining for Nodal and Notch4 was significantly more common in advanced stage (stage III–IV) melanomas than in early stage (stage I–II) melanomas (64% for Nodal and 40% for Notch4 in stage III–IV (n = 25) vs. 36% for Nodal and 17% for Notch4 in stage I–II (n = 36); P < 0.05; Fig. 2E; Supplementary Fig. 2; Supplementary Table 3). Altogether, these data indicate a specific Notch4 and Nodal correlation both in aggressive melanoma cell subpopulations and in advanced stage melanomas.

**Notch4 activity is required for Nodal expression in aggressive melanoma cell lines**

To test the hypothesis that Notch4 signaling modulates Nodal expression, C8161 and MV3 cells were transfected with small inhibitory RNAs (siRNAs) to each Notch receptor, and Nodal expression was assessed by Western blotting (Fig. 3A). siRNA specificity was verified by real-time PCR (Supplementary Table 4) and confirmed for Notch4 by Western blotting (Supplementary Fig. 3). Cells transfected with siRNA to Notch1 (siNotch1), Notch2 (siNotch2), Notch3 (siNotch3; shown for MV3), or negative control (siCON) had little effect on Nodal expression in either cell line (Fig. 3A). In contrast, siRNA to Notch4 (siNotch4) significantly reduced expression of Nodal protein (C8161, 62% ± 1%; MV3, 49% ± 4%; P < 0.05). In addition, Notch4 activity in C8161, MV3, and SK-MEL-28 cells was antagonized with a previously characterized anti-human Notch4 neutralizing antibody (24) and Nodal expression was examined by real-time PCR (Fig. 3B) and Western blotting (Fig. 3C). Treatment with anti-Notch4 antibody dramatically reduced Nodal transcripts in all cell lines compared with IgG-treated control cultures (Fig. 3B; P < 0.05). Similarly, Nodal protein levels were highly downregulated in anti-Notch4–treated cultures (C8161, 73% ± 8%; MV3, 71% ± 2%; SK-MEL-28, 73% ± 5%; P < 0.05; Fig. 3C).

To show anti-human Notch4 antibody specificity, C8161 cells were transfected with a Notch4–ICD expression vector and then antagonized with the anti-Notch4 antibody or goat IgG (Supplementary Fig. 4A). IgG-exposed, untransfected and transfected Notch4–ICD cells expressed typical Nodal protein levels. However, untransfected cells exposed to anti-Notch4 antibody showed practically no Nodal protein whereas antibody-treated cells expressing Notch4–ICD recovered expression of Nodal protein. This finding suggests that Notch4–ICD regulates some Nodal expression. Further validation of the Notch4–Nodal relationship was discovered in poorly aggressive UACC1273 cells (that typically lack Nodal) transfected with Notch4–ICD, which resulted in Nodal upregulation (Supplementary Fig. 4B). Collectively, these data strongly support a role for Notch4 activity in upregulating Nodal in melanoma cell lines.

**Notch4 inhibition impacts cellular proliferation and apoptosis**

To determine the effects of inhibiting Notch4 signaling on cellular growth, C8161, MV3, and SK-MEL-28 cells treated with anti-Notch4 antibody or IgG were analyzed for cell population...
Figure 2. Survey of Notch receptors and Nodal expression in melanoma cell lines. A, RNA isolated from 2 poorly aggressive cell lines (UACC1273 and c81-61) and 4 aggressive cell lines (C8161, MV3, SK-MEL-28, and WM852) was assayed for gene expression of Notch1–4 and Nodal by semiquantitative PCR. GAPDH was a loading control. DNA contamination was excluded using no MMLV (not shown). B, protein lysates were analyzed for Notch receptor and Nodal proteins by Western blotting. Actin was a loading control. C, Nodal (green) and Notch4 (red) proteins were examined by confocal microscopy in C8161, MV3, and SK-MEL-28 cells (also Supplementary Fig. 1). Arrowheads in inset (*) denote regions of colocalization (yellow). DAPI (blue) marks cell nuclei. White bar represents 10 μm. D, cells positive for Nodal, Notch4, or both Nodal and Notch4 (Nodal + Notch4) were independently counted using a 25× objective. For each category, mean ± SD was graphed as a percentage of total DAPI-positive nuclei (n = 7). E, immunohistochemistry of Nodal and Notch4 on serial sections of a human melanoma tissue array. The number of tissue samples showing strong staining (>50%) was graphed as a percentage of total samples evaluated (for stage I–II, n = 36; for stage III–IV, n = 25). *, P < 0.05.
size (Fig. 4A), cell viability (Fig. 4B), and apoptosis (Fig. 4C) by flow cytometry. Compared with IgG cell populations that typically doubled over the culture period, anti-Notch4–treated cells showed little proliferative increase and remained significantly lower than control cell population size (Fig. 4A; \( P < 0.05 \)). In contrast, control cells exhibited consistently high viability whereas anti-Notch4–treated cells displayed a significant viability decline (Fig. 4B; \( P < 0.05 \)). Furthermore, the
Figure 4. Inhibition of Notch4 activity limits cell proliferation and promotes apoptosis. C8161, MV3, and SK-MEL-28 cells were treated with anti-human Notch4 antibody or IgG. A–C, at 24-hour time points, cells were assayed for cell number (A), viability (B), and apoptosis (C) by flow cytometry. Cell number is shown as a percentage of the initial population, whereas viability and apoptosis are represented as the percentage of total cells. Plots represent the mean ± SD of 3 independent experiments done in triplicate; *, P < 0.05. D, Western blot analyses of Histone H3 phosphorylation and PCNA expression (proliferation), and PARP cleavage (apoptosis) in C8161, MV3, and SK-MEL-28 cells. Actin was a loading control. Membranes were stripped between antibody detections. Western blots are representative of 3 experiments.
percentage of apoptotic cells remained constantly low in controls but dramatically increased in anti-Notch4–treated cultures (Fig. 4C; \( P < 0.05 \)).

To complement this approach, the same 3 cell lines were antibody treated and evaluated for markers of proliferation [HistoneH3 phosphorylation and proliferating cell nuclear antigen (PCNA) expression] and apoptosis [poly-ADP-ribose polymerase (PARP) cleavage] by Western blotting (Fig. 4D). Phospho-HistoneH3 was detected in untreated and IgG-treated cells but was dramatically reduced in anti-Notch4–treated cells despite similar total HistoneH3 levels. PCNA expression was also reduced in anti-Notch4–treated cells compared with untreated and IgG-treated cells. In contrast, cleaved PARP was detected in anti-Notch4–treated cultures but not in untreated or IgG-treated cultures whereas full-length PARP expression levels were similar or reduced in anti-Notch4–treated cells. Collectively, these findings indicate that Notch4 activity modulates cell proliferation and survival in aggressive melanoma cell lines.

**Inhibition of Notch4 activity impairs vasculogenic mimicry and diminishes clonogenicity in vitro in a Notch4-dependent manner**

We next addressed the role of Notch4 in regulating melanoma cell behavior. C8161 and other aggressive melanoma cells typically engage in vasculogenic mimicry when grown on 3D-collagen matrix (25, 28–30). Notch4 is expressed in endothelial cells and functions in vascular formation and remodeling, as well as in tumor angiogenesis (9, 31–33). Considering the relationship between tumor angiogenesis (recruitment of new vessels into a tumor from existing vessels) and tumor vasculogenic mimicry (de novo formation of vascular-like networks by non–endothelial tumor cells), we reasoned that Notch4 may also participate in vasculogenic mimicry. The ability of C8161 and SK-MEL-28 cells to form vascular-like network structures without Notch4 activity was evaluated in vitro (Fig. 5A). Compared with untreated (left) and IgG cultures (middle left), in which vascular-like networks formed (white arrows), anti-Notch4 antibody treatment severely impaired vascular-like network formation (middle right). When cultures exposed to anti-Notch4 antibodies were treated with recombinant human Nodal protein, vascular-like network formation was restored (right). Recombinant Nodal also rescued VE-cadherin and endogenous Nodal expression downregulated by Notch4 inhibition in C8161 cells (Supplementary Fig. 5A). Anti-Notch4–treated C8161 cells expressing Notch4–ICD also recovered Nodal and VE-cadherin protein expression (Supplementary Fig. 5B). These data indicate that Notch4 regulation of vasculogenic mimicry is likely Notch4-dependent.

The tumorigenic potential of Notch4 function was evaluated via in vitro clonogenic assays. C8161 and SK-MEL-28 cells pretreated with anti-Notch4 antibody formed significantly fewer macroscopic colonies in soft agar than untreated or IgG-pretreated cells (Fig. 5B and C; \( P < 0.05 \)). Notably, cells pretreated with anti-Notch4 antibody plus recombinant Nodal protein recovered some anchorage-independent growth capacity (\( P < 0.05 \)). Altogether, these data suggest that Notch4 functions to promote the tumorigenic phenotype in vitro, likely in part through regulation of Nodal expression.

**Discussion**

The aberrant reexpression of Nodal in metastatic melanoma cells critically regulates cellular plasticity and aggressiveness (2, 3). Here, we identify a decisive link between Notch4 and Nodal in multiple aggressive melanoma cell lines that modulates the aggressive tumorigenic phenotype in vitro, in part through regulation of Nodal. We describe expression of Nodal and Notch4 protein in advanced stage human melanomas, which further validates a recent study of Nodal in melanocytic lesions (8). Our observations are confirmed by earlier work in mouse xenograft models (3, 34). In one study, a synthetic \( \gamma \)-secretase inhibitor (GSI), which indiscriminately inhibits Notch receptor activation, significantly diminished in vivo melanoma tumor volume in nude mice injected with C8161 cells (34). Whether the observed reduction in tumor growth was coincident with downregulation of Nodal was not evaluated but would offer an interesting explanation for the observed effect considering that Nodal has also previously been shown to regulate in vivo melanoma tumor formation in xenografted nude mice (3). Considering the evidence presented in our current study, we speculate that inhibition of Notch4 activity might similarly affect in vivo melanoma tumor formation.

We describe the coexpression of Nodal and Notch4 in subpopulations of aggressive melanoma cells and the requirement of Notch4 for Nodal expression. We also show that Nodal expression is upregulated by exogenously expressed Notch4–ICD. Together these data provide strong indication that Notch4 lies upstream of Nodal expression in, at least, the aggressive tumor cell lines studied herein. While, by immunofluorescence microscopy, some cells express both Notch4 and Nodal, the observation that select cells express one protein but not the other is likely indicative of the dynamic nature of this cancer cell population. Considering that Nodal can function in a paracrine fashion, protein observed in some cells may reflect uptake from the microenvironment rather than intracellular production. Nodal expression is maintained on a feed-forward loop, so Notch4 signaling may be required for initial Nodal expression in a cell but then be dispensable for its maintenance. Notch4 expression independent of Nodal may simply reflect early-stage signaling before convergence on the Nodal gene. However, it is possible that other factors also contribute to the regulation of Nodal.

Notch4 signaling is required for cell proliferation and survival in aggressive melanoma cells and also modulates cellular plasticity and tumorigenic growth in vitro. Of significance, some phenotypic effects can be attributed to Notch4 regulation of the Nodal gene, as treatment with recombinant human Nodal rescues vasculogenic mimicry and anchorage-independent growth in vitro. However, recombinant human Nodal could not rescue cell growth in flow cytometry assays (data not shown), perhaps because of the shorter length of these experiments relative to other
assays or because Notch4 function in cell growth is independent of Nodal.

As both the Notch and Nodal pathways are involved in stem and progenitor cell maintenance (35–38), we speculate that the subpopulation of cells expressing both Notch4 and Nodal may retain special properties such as enhanced cellular plasticity. Certainly, Notch4 activity regulates plasticity required for vasculogenic mimicry and anchorage-independent growth, though whether this plasticity is driven by a subpopulation of cells is not clear. One recent study specifically linked Notch4 activity to a breast cancer stem cell (BCSC) subpopulation capable of \textit{in vivo} tumor formation (39). That study described higher Notch4 activity than Notch1 activity in the BCSC subpopulation.

Targeting Notch4 expression more effectively reduced the BCSC subpopulation and inhibited \textit{in vivo} tumor formation than targeting Notch1, suggesting, at least in breast cancer, that only specific Notch receptors promote cancer stem cell phenotypes. This may also hold significance for melanoma.

No role for Notch4 signaling has previously been described in melanoma, though other studies have linked Notch1 with melanoma disease progression and the transformation of melanocytes or early-stage melanoma cells to a more aggressive phenotype (5, 40–42).

In some contexts, such as in embryonic development and in MMTV-mediated mammary gland tumor formation, Notch receptors have common functions (32, 43–45). However, Notch receptors have structural differences (Notch4 lacks TAD and

Figure 5. Inhibition of Notch4 signaling blocks vasculogenic mimicry and anchorage-independent growth \textit{in vitro} in a Nodal-dependent manner. A, C8161 and SK-MEL-28 cells were seeded on 3D-collagen gel matrix and left untreated or treated with IgG, anti-Notch4 antibody, or anti-Notch4 antibody plus recombinant human Nodal (rNodal). White arrows indicate vascular-like network formation in untreated (far left), IgG-treated (center left), and anti-Notch4 + rNodal-treated cultures (far right). Original magnification \times 100. B and C, relative colony formation in C8161 (B) and SK-MEL-28 (C) cells cultured on soft agar following pretreatment with IgG or anti-Notch4 antibody with or without rNodal. Graph indicates macroscopic colonies as a percentage (mean \pm SD) of control.* \textit{vs} significant difference from untreated/IgG-treated cultures (\(P < 0.05\)); ** \textit{vs} significant difference from anti-Notch4–treated cultures (\(P < 0.05\)). Graphs depict 1 of 3 representative experiments.
NCR domains near the C-terminus and some epidermal growth factor repeats near the N-terminus) that may be significant (9, 10) and studies have shown functional diversity among the Notch receptors (46–48). The present study indicates an independent function for Notch4 in regulating the Nodal gene in melanoma. Other work illustrates that Notch4 functions uniquely in breast cancer (39). Recently, Pinnix et al. showed increased Notch4 mRNA in melanoma cell lines and patient tissue lesions compared with cultured melanocytes (5). However, inconsistent with our study, they observed similar levels of Notch4 mRNA in c81-61 (poorly aggressive) and C8161 (aggressive) cells. It is possible that "late passage" c81-61 cells utilized by Pinnix et al., as opposed to early passage c81-61 cells used in our study, have acquired some aggressive phenotypic markers (including Notch4 expression) consistent with genetic and phenotypic drift commonly seen with long-term in vitro culture conditions (49, 50). It is likely that molecular cross talk between Notch and Nodal in melanoma is complex, as we fully appreciate that our own study examined only 6 human melanoma cell lines with respect to this relationship. It will be important for subsequent studies to extend our findings in additional cell lines and learn whether other Notch receptors can regulate Nodal expression.

Notch4 is involved in vessel patterning and remodeling during development (32, 33) and adulthood (9), and participates in tumor angiogenesis (31) and vasculogenic mimicry (as described herein). On the basis of our validation of Nodal and Notch4 coexpression in advanced stage disease, targeting Notch4 in melanoma may offer an attractive 2-hit therapeutic strategy, by impairing tumor cell plasticity and limiting tumor microcirculation and growth. Understanding regulatory mechanisms that promote the aggressive phenotype, such as the Notch4–Nodal relationship, may provide useful exploratory avenues for melanoma prevention and treatment strategies.

Disclosure of Potential Conflicts of Interest

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Regulation of the Embryonic Morphogen Nodal by Notch4 Facilitates Manifestation of the Aggressive Melanoma Phenotype

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