A Role for Jag2 in Promoting Uveal Melanoma Dissemination and Growth

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PURPOSE. Controlling the spread of uveal melanoma is key to improving survival of patients with this common intraocular malignancy. The Notch ligand Jag2 has been shown to be upregulated in primary tumors that metastasize, and we therefore investigated its role in promoting invasion and clonogenic growth of uveal melanoma cells.

METHODS. mRNA and protein expression of Notch pathway components were measured using qPCR and Western blot in uveal melanoma cell lines. Expression of Jag2 ligand was upregulated using Jag2-GFP-MSCV constructs or downregulated by sh-Jag2 in the uveal melanoma cell lines Mel285, Mel290, 92.1, and OMM1, and the effects on growth and invasion were assessed.

RESULTS. Jag2 was introduced into Mel285 and Mel290 cells, which have low baseline levels of both this ligand and Notch activity. Overall growth of the Jag2-expressing cultures increased somewhat, and a significant 3-fold increase in clonogenic growth in soft agar was also noted. Introduction of Jag2 increased motility in both wound-healing and transwell invasion assays. We also observed a significant increase in Jag2 and Hes1 mRNA in invasive OMM1 cells that had passed through a Matrigel-coated filter in the transwell assay when compared with noninvading cells. Loss-of-function studies performed in 92.1 and OMM1 lines using Jag2 shRNAs showed that downregulation of the ligand significantly suppressed cellular growth, invasion, and migration.

CONCLUSIONS. Our data suggest that Jag2 may play an important role in promoting Notch activity, growth, and metastasis in uveal melanoma. (*Invest Ophthalmol Vis Sci.* 2013;54:295–306) DOI:10.1167/iovs.12-10209

The eye is the second most common site for primary melanoma after the skin, accounting for 5% of all melanomas.¹ Melanomas arising in the uvea represent the

most frequent primary intraocular malignancy in adults. Uveal melanomas have a strong propensity to metastasize by hematogenous dissemination, with a preferential tropism for the liver (93%), but sporadic metastases are also found in the lung (24%) and in the bone (16%).² The cause of this selective hepatic dissemination is still unclear. The mortality rate 15 years after diagnosis of the primary tumor is approximately 50%, with a median survival time of 6 to 9 months after detection of hepatic metastasis.³ Despite advances in the treatment of primary tumors through episcleral brachytherapy, transpupillary thermotherapy, and local surgical resection, no effective therapies have been developed that prevent or treat metastatic disease.^{4,5} A better understanding of the mechanisms responsible for metastatic spread of primary uveal melanoma is therefore necessary to find new therapeutic approaches to prevent tumor dissemination and growth of metastatic lesions.

Specific chromosomal abnormalities are linked to tumor progression and poor prognosis in uveal melanoma. One of the most important alterations is loss of one copy of chromosome 3, which occurs in almost half of the primary tumors and is considered the most significant chromosomal marker of poor outcome in uveal melanoma.⁶ Other chromosomal alterations previously associated with outcome include 6p gain, which protects against metastatic spread, and 8p loss, which portends poor prognosis.^{7,8} In addition, inactivating mutation of the tumor suppressor BRCA1-associated protein-1 (*BAP1*), located at 3p21.1, has been found exclusively in metastatic uveal melanomas with monosomy 3 and is thought to play a fundamental role in promoting tumor metastasis.⁹ Although *BAP1* mutations are thought to occur late during tumor progression, oncogenic mutations in the subunit α of a stimulatory G protein (*GNAQ*^{Q209L} or its paralog *GNAI1*^{Q209L}), which activates RAF/MEK/ERK signaling, are considered early events and were found in more than 80% of uveal melanomas.^{10,11}

Recently, gene expression profiling has allowed classification of primary uveal melanomas into prognostically significant groups. Studies by several groups have identified differences between class 1 tumors, with low metastatic risk, and class 2 tumors, which are more prone to metastatic dissemination.^{4,12,13} Class 1 tumors retain a differentiated, melanocytic transcriptional profile, whereas class 2 tumors exhibit a stem-like transcriptome and often contain cells that have lost a copy of chromosome 3. Others have used multiplex ligation-dependent probe amplification to detect chromosomal abnormalities in uveal melanomas as another method to determine clinical outcome.¹⁴

In analyzing published expression data, we previously reported that levels of the Notch ligand Jag2 were elevated 1.9-fold in uveal melanoma, which metastasized.¹⁵ In the same report, we confirmed high levels of expression of Jag2 mRNA and other Notch pathway members in the majority of 30 additional snap-frozen tumor samples compared with nonneoplastic melanocytes. Although we were not able to make

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associations with outcome in the smaller cohort due to a lack of available clinical follow up, this led us to investigate the role of Notch signaling in these tumors.

We found that canonical Notch signaling plays an important role in promoting tumor growth and invasion in human uveal melanoma cultures.¹⁵ The Notch pathway controls stem cell self-renewal and differentiation in many organs, including retinal pigment epithelial and retinal ganglion cells.^{16,17} The pathway includes four receptors (Notch1–4) and five ligands: DLL1,3,4 (Delta-like ligands) and Jagged1–2 (Jag1–2). Ligand binding activates two successive proteolytic cleavages, the latter being mediated by the enzyme γ -secretase, with consequent release of the intracellular portion of the Notch receptor (ICN) in the nucleus, where it forms a complex with several cofactors, including CBF1 and MAML. This heteromeric complex activates the transcription of the Notch target genes, including the Hairy and enhancer of split (Hes) and Hes-related repressor protein families.¹⁸

Although our prior work suggests that Notch signaling promotes uveal melanoma growth, the mechanism by which the pathway is activated remains unclear. Point mutations can activate Notch in a ligand-independent fashion in leukemic cells, but such mutations were not identified in a recent comprehensive sequence analysis of uveal melanoma^{9,19}; therefore, we examined the possibility that increased expression of pathway ligands might promote Notch activity and aggressive behavior in uveal melanoma.

METHODS

Cell Lines and Plasmids

Five cell lines derived from primary human choroidal melanoma: OCM1,²⁰ OCM3,²¹ Mel285,²² Mel290,²² 92.1,²³ and one line obtained from a subcutaneous metastasis, OMM1,²⁴ were kindly provided by Dr. Jerry Y. Niederkorn (UT Southwestern Medical Center, Dallas, TX), and cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (2 mM), HEPES buffer (10 mM), sodium pyruvate (1 mM), MEM essential vitamin mixture (1% solution), MEM nonessential amino acid (1% solution), 50 IU/mL penicillin, and 50 μ g/mL streptomycin at 37°C. With local Institutional Research Board approval, tumor tissue was isolated from primary uveal melanomas removed by enucleation at the Wilmer Eye Institute from 2004 to 2006 and snap frozen. Retroviruses were generated as previously described,²⁵ using human Jag2-GFP-MSCV and GFP-MSCV constructs kindly provided by Drs. Jason Yustein²⁶ and Nadia Barlesso (Indiana University, Bloomington, IN). Lentiviruses encoding small hairpin RNAs (shRNAs) against Jag2 (sequence no. 1: CTC TCA CAC AAA TTC ACC AAA; sequence no. 2: GTC GTA CTT GCA CTC ACA ATA) or CBF1 (sequence no. 2: CCC TAA CGA ATC AAA CAC AAA; sequence no. 3: GCA CAG ATA AGG CAG AGT ATA; Thermo Fisher Scientific, Huntsville, AL) were prepared in PLKO.1 as previously described.²⁷ Gamma-secretase inhibitor (GSI) MRK003 was obtained commercially (Merck & Co., Inc., Whitehouse Station, NJ).²⁸

RNA Extraction and Quantitative Real-Time PCR

RNA was extracted from cell lines using a commercial kit (RNeasy Mini Kit; Qiagen, Germantown, MD) with on-column DNA digestion. Quantitative real-time PCR (qPCR) was performed as previously described,²⁹ using primer sequences already published.¹⁵ All reactions were repeated in triplicate using a real-time PCR detection system (iQ5 multicolor detection system; Bio-Rad, Hercules, CA), using SYBR Green (Applied Biosystems, Foster City, CA) fluorescent dye, and normalized to β -actin mRNA levels.

To separate cells with different invasion rate, 5×10^5 cells were plated in 25-mm-diameter transwell cell culture inserts (Falcon inserts, 8- μ m pore size; Becton Dickinson, Franklin Lakes, NJ) precoated with a biodegradable gelatinous protein mixture secreted by mouse tumor cells (Matrigel, diluted 1:100 in 10% FBS medium; BD Biosciences, Franklin Lakes, NJ) in six-well plates. After overnight incubation, cells that had migrated on the bottom of the filter were lysed and the RNA was extracted using a commercial kit (RNeasy Mini Kit; Qiagen). The noninvading cells on the top of the filter were removed with a cotton swab. The opposite procedure was used in an adjacent well containing the same number of cells, to collect RNA from noninvading cells. The experiment was performed in triplicate, using three wells for the invading and three for the noninvading cells.

Protein Analysis

Cells were lysed in TNE buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 5 mM EDTA; 1% SDS) supplemented with protease inhibitor cocktail diluted 1:100 (Cytoskeleton Inc., Denver, CO) and 1 mM sodium orthovanadate. The lysates were sonicated on ice for 20 seconds. Protein concentration was measured using a bicinchoninic acid (BCA) assay kit (BCA Protein Assay Kit; Thermo Fisher Scientific/Pierce Protein Biology Products, Rockford, IL). Equivalent amounts of proteins were analyzed by SDS-polyacrylamide gel electrophoresis. After electrophoretic separation, proteins were transferred onto nitrocellulose membrane (Invitrogen) and incubated overnight at 4°C with the following antibodies: Jag2, 1:800 (#2210; Cell Signaling Technology, Danvers, MA), GAPDH, 1:5000 (#TRK5G4-6C5; RDI, Flanders, NJ), phospho-Erk1–2^{Thr202/Tyr204}, phospho-Akt^{Ser473}, Erk1–2, Akt (Cell Signaling Technology), all diluted 1:1000. Proteins were visualized with peroxidase-coupled secondary antibodies (KPL, Gaithersburg, MD), using enhanced chemiluminescence (ECL) for detection (PerkinElmer, Waltham, MA).

Cell Growth, Invasion, and Migration Assays

Cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) colorimetric assay, as previously described.³⁰ For anchorage-independent growth, 1×10^4 cells were mixed with medium containing 0.5% agar (Invitrogen) and placed over 1% basal agar in six-well plates. Colonies were counted with a commercial colony counter (Artek Counter Model 880; Artek Systems Corp., Farmingdale, NY), as previously described.¹⁵

Cellular invasion experiments were performed using transwell assays. Briefly, 6.5-mm-diameter cell culture inserts (Falcon insert, 8- μ m pore size; Becton Dickinson) were precoated for 1 hour (Matrigel, diluted 1:100 in 10% FBS medium) in 24-well plates. Cells were trypsinized and resuspended in serum-free RPMI 1640 medium in the upper chamber of the filter (10^5 cells in 500 μ L); 800 μ L of medium containing 10% FBS were added in the lower chamber, and after overnight incubation, cells remaining on the upper surface of the filter were removed with a cotton swab. Cells that had migrated to the lower surface were fixed with ethanol, stained with hematoxylin, destained, and photographed. Data represent the mean $\pm$ SEM of the number of cells counted in six random high-power fields (HPFs) in each of three independent experiments.

The migration properties of the cells were evaluated using both transwell and wound-healing assays. The transwell migration assay was carried out using 6.5-mm-diameter cell culture inserts (Falcon insert, 8- μ m pore size; Becton Dickinson) precoated with 0.1% gelatin in 10% FBS medium. For the wound-healing assay, 1.5×10^5 cells were seeded in 12-well plates and incubated overnight at 37°C to generate confluent cultures. After achieving confluence, the cellular layer in each well was scratched using a plastic pipette tip. The migration of the cells at the edge of the scratch was monitored at time 0 and 16 hours, when cells

were stained with crystal violet for 10 minutes, destained in PBS, and visualized microscopically.

Statistical Analysis

Experiments were performed in triplicate and data are presented as the mean + SEM. Levels of significance were determined by two-sided Student's *t*-test, with values of $P < 0.05$ considered statistically significant. Statistical calculations were performed using commercial software (Prism4 software; GraphPad, San Diego, CA).

RESULTS

Jag1 and Jag2 Notch Ligands Are Expressed in Uveal Melanoma Cell Lines

We analyzed by qPCR the expression of Notch pathway ligands and target genes in six established uveal melanoma cell lines, and found that Jag1 ligand mRNA was expressed at varying levels in all of these, although expression was low in Mel290 and OMM1. In contrast, Jag2 mRNA was elevated only in OMM1 and 92.1 cells (Fig. 1A). Relatively high Notch activity was also noted in OMM1 and 92.1 cells, as evidenced by increased Hes1 and Hey1 levels. The expression of Jag2 at the protein level in OMM1 and 92.1 cells was similar to that observed in primary uveal melanoma samples (Fig. 1B).

We had previously shown that OCM1 and OCM3 cells with elevated Hes1 and Hey1 mRNA expression had their growth suppressed following Notch blockade using the gamma-secretase inhibitor MRK003, whereas lines with low baseline Notch activity (Mel285, Mel290) were relatively resistant.¹⁵ We had not previously examined the requirement for Notch activity in OMM1 and 92.1 cells, which feature activating mutations in *GNAQ* and *GNA11*, respectively, in contrast to the lines that we previously examined that lack genetic changes at these loci.³¹ Despite these genetic differences, we found that MRK003 treatment also significantly slowed the growth of OMM1 and 92.1 cells (Fig. 1C).

To test our central hypothesis that Jag2 promotes the efficient metastatic spread of uveal melanoma, we analyzed the mRNA levels of Jag2 and Hes1 in OMM1 cells that had migrated overnight through a microporous membrane precoated with Matrigel, which mimics the composition and properties of the extracellular matrix (Fig. 1D). Interestingly we found that both Jag2 and the Notch pathway target Hes1 were significantly increased in the invasive cells that had migrated to the lower surface of the membrane (bottom), compared with those that did not move through the filter (top). Whereas Mel290 cells have low overall levels of Jag2 and Hes1 expression, both of these were also significantly higher in cells that invade through Matrigel (data not shown). These findings suggest that Jag2 might drive invasion in uveal melanoma.

Increased Jag2 Expression Enhances Cell Growth, Invasion, and Migration in Mel285 and Mel290 Cells

Because both Jag2 expression and Notch activity were low in Mel285 and Mel290 cultures, we tested the effect of increased ligand expression in these lines. Constructs encoding human Jag2 ligand were introduced through retroviral infection into Mel285 and Mel290 cells, using cells infected with MSCV vector alone as controls. The Jag2-MSCV-infected cells showed a massive increase in Jag2 mRNA levels, along with a 10- to 16-fold induction of the Notch target Hes1 (Figs. 2A, 2B). This indicates that the Notch pathway is capable of further

activation in the Mel285 and Mel290 lines, and suggests that ligand levels represent a limiting factor.

Jag2 also had several significant functional effects. Although upregulation of Jag2 caused only a modest increase in cell proliferation in adherent conditions, as determined by MTS assay (Figs. 2C, 2D), a more profound growth advantage was observed in anchorage-independent conditions, where Jag2-MSCV-infected cells showed an approximately 3-fold increase in clonogenic growth in soft agar (Fig. 2E). We also analyzed the effects of Jag2 on cellular invasion and migration. Overexpression of Jag2 ligand induced a significant ($P < 0.0003$) 2-fold increase in the transwell invasion capacity of Mel285 and Mel290 cells through microporous membranes precoated with Matrigel (Figs. 3A, 3B). Jag2-MSCV-infected cells also increased their ability to migrate compared with control cells, as determined by wound-healing ("scratch") assay in Mel290 and by transwell migration assay in Mel285 cells (Figs. 3C, 3D).

Jag2 Effects Are Mediated by Canonical Notch Signaling

Whereas Jagged ligands are thought to act primarily through activation of Notch receptors, noncanonical effects have been reported.³²⁻³⁴ To elucidate whether the effects induced by Jag2 in uveal melanoma cells are due to Notch signaling, we suppressed expression of the canonical transcriptional cofactor CBF1 using shRNAs in Jag2-MSCV-Mel290 cells, and analyzed whether the increase in cell proliferation and invasion following Jag2 upregulation was abrogated. Two previously validated target sequences were used¹⁵ and both of them significantly reduced CBF1, Hey1, and Hey2 mRNA levels in Jag2-MSCV-Mel290 cells, compared with scrambled shRNA (Fig. 4A). We observed that the increases in cell proliferation and invasion following upregulation of Jag2 were significantly reduced by shRNA targeting CBF1 (Figs. 4B, 4C), suggesting that the effects due to Jag2 overexpression are mediated at least in part by canonical Notch activity. To further investigate the hypothesis that canonical Notch activity is implicated in promoting ligand-dependent growth and invasion in uveal melanoma, we suppressed CBF1 expression with sh-CBF1 in 92.1 cells that express high endogenous levels of Jag2 (Fig. 5A). We observed that such reduction correlated with a significant decrease in cellular proliferation and invasion (Figs. 5B, 5C).

Downregulation of Jag2 Reduces Cell Growth, Invasion, and Migration in OMM1 and 92.1 Cells

To further examine the role of Jag2 in the regulation of uveal melanoma growth and invasion, we performed loss-of-function studies with shRNAs targeting Jag2 in OMM1 and 92.1 cells, where the endogenous levels of the ligand are high compared with the other uveal melanoma lines that we analyzed (Figs. 1A, 1B). Two separate constructs significantly reduced Jag2 mRNA and protein expression, as well as Hes1 and Hey1 mRNA levels in both cell lines (Figs. 6A, 6B), suggesting that other Notch ligands cannot substitute for the loss of Jag2 in these cells. Knock down of Jag2 expression inhibited cell growth in adherent conditions (Figs. 6C, 6D). Colony formation in soft agar was also significantly reduced by both sh-Jag2 constructs in 92.1 cells, whereas in OMM1 cells only target sequence #2 suppressed clonogenic growth (data not shown). Furthermore, a significant ($P < 0.0001$) decrease in cellular invasion into Matrigel was found using the transwell assay (Figs. 7A, 7B) in OMM1 and 92.1 cells infected with sh-Jag2. The ability of the cells to migrate was also drastically impaired by inhibiting Jag2 expression in 92.1 cells, as

FIGURE 1. Expression of Notch pathway ligands and target genes in uveal melanoma cell lines. (A) mRNA levels of Jag1, Jag2, Hes1, and Hey1 were analyzed by quantitative PCR in six uveal melanoma cell lines. (B) Jag2 protein expression was determined in six primary tumors (*left panel*) and in six uveal melanoma lines (*right panel*). (C) MTS assay in OMM1 and 92.1 cells treated with MRK003 at the indicated doses for 7 days reveals that cellular growth in both lines is sensitive to Notch inhibition. (D) Jag2 and Hes1 mRNA levels are increased in OMM1 cells that passed through a gelatinous protein mixture (Matrigel)-coated filter (*bottom*) compared with noninvading cells (*top*).

FIGURE 2. Upregulation of Jag2 increases cell growth. (A, B) Jag2 and Hes1 mRNA levels are induced after infecting Mel290 (A) and Mel285 (B) cells with Jag2-MSCV vector compared with MSCV control. (C, D) MTS assay in Mel290 (C) and Mel285 (D) cells shows that cellular growth is significantly increased in Jag2-MSCV-infected cells compared with vector control. (E) Clonogenic growth in soft agar is increased in Mel290 cells infected with Jag-MSCV compared with MSCV-infected cells. Microphotographs in the right panel show the colony morphology.

FIGURE 3. Jag2 overexpression increases cellular invasion and migration. (A) Transwell invasion assay reveals a significant increase in the invasion capacity of Mel290 (A) and Mel285 (B) cells overexpressing Jag2, compared with vector control-infected cells. Microphotographs (*bottom panels*) show the invading cells on the lower surface of the protein mixture (Matrigel)-coated filter after 16 hours of incubation. (C) Wound-healing assay shows an increase in cell migration in Jag2-MSCV Mel290 cells compared with MSCV control. (D) Transwell migration assay performed in Mel285 cells infected with Jag2-MSCV indicates a significant increase in cellular migration in the cells that overexpress Jag2. The *lower panel* shows the morphology of the migrated cells after overnight incubation.

FIGURE 4. Inhibition of canonical Notch signaling reduces the effects due to the upregulation of Jag2. (A) CBF1 (*left*) and Hey1, Hey2 mRNA levels (*right*) are reduced in Jag2-MSCV Mel290 cells infected with two separate sh-CBF1 constructs (no. 2, no. 3) compared with cells infected with scramble shRNA. (B) MTS assay after 7 days of incubation shows that the induction in cell growth due to Jag2 upregulation in Mel290 cells is partially suppressed by inhibiting CBF1 expression. (C) Transwell invasion assay indicates that the increase in cellular invasion observed in Jag2-MSCV Mel290 cells is totally suppressed when the cells are infected with sh-CBF1 compared with scramble control shRNA. Microphotographs in the *bottom panels* show the invading cells on the lower surface of the protein mixture (Matrigel)-coated filter after 16 hours of incubation.

FIGURE 5. Inhibition of CBF1 suppresses growth and invasion in 92.1 cells. **(A)** CBF1 mRNA levels are reduced in 92.1 cells infected with sh-CBF1 constructs (no. 2, no. 3) compared with scramble shRNA. **(B)** MTS assay shows that the repression of CBF1 inhibits cell growth. **(C)** Transwell invasion ability of 92.1 cells is impaired by reducing CBF1 expression. Microphotographs in the *right panels* show the invading cells on the lower surface of the protein mixture (Matrigel)-coated filter after 24 hours of incubation.

determined by transwell migration over a gelatin substrate (Fig. 7C) and in wound healing assays (Fig. 7D).

Jag2 Expression Increases Erk1–2 Phosphorylation

To investigate the mechanism responsible for the increase in cell proliferation and invasion induced by Jag2 ligand, we analyzed the phosphorylation status of Erk1–2 in Mel290 and Mel285 cells overexpressing Jag2. Intriguingly, we observed a substantial increase in Erk1–2 phosphorylation in both cell lines where Jag2 was upregulated compared with MSCV-infected cells (see Supplementary Material and Supplementary Figs. S1A, S1B, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10209/-DCSupplemental>). However no difference was observed in Akt phosphorylation (data not shown). In addition, Erk1–2 phosphorylation was reduced in OMM1 cells infected with sh-Jag2 (see Supplementary Material and Supplementary Fig. S1C, <http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-10209/-DCSupplemental>), where we also observed a strong reduction in cell growth (Fig. 6C), suggesting that the positive effects of Jag2 on cell proliferation might be due to the activation of Erk1–2.

DISCUSSION

A number of findings have led us to focus on the potential role of the Notch pathway in uveal melanoma growth and dissemination. First, Notch is known to play a role in the initiation and growth of cutaneous melanoma.^{35,36} Second, we have previously shown that introduction of constitutively active Notch3 can induce the formation of pigmented, invasive uveal tumors in mice.³⁷ Third, in analyzing genes associated with the class two expression signature of uveal melanoma, we found that elevated levels of several Notch ligands and receptors, including Jag2, were present in metastasis-prone tumors.¹⁵ In this previous study, we confirmed that the Notch pathway is active in many primary uveal melanomas, but were not able to further investigate its prognostic role in the small independent cohort due to a lack of follow-up data. We did demonstrate that Notch signaling can induce proliferation and invasion in uveal melanoma cultures, but the mechanism by which the pathway was activated was not clear.¹⁵ Here, we focused on the potential role of the ligand Jag2 in promoting Notch pathway activity, cellular invasion, and tumor growth in uveal melanoma.

In the current study, we observed that Jag2 protein is expressed in primary uveal melanoma lysates, and that the

FIGURE 6. Downregulation of Jag2 reduces cell growth in OMM1 and 92.1 cells. (A, B) Jag2, Hes1, Hey1 mRNA levels are reduced in OMM1 (A) and 92.1 (B) cells infected with two different sh-Jag2 constructs. *Right panels* show the reduction in Jag2 protein levels in OMM1 and 92.1 cells infected with sh-Jag2 compared with scramble shRNA. (C, D) Cellular growth in OMM1 (C) and 92.1 (D) cells is significantly reduced by inhibiting Jag2 expression as determined by MTS assay.

highest levels in these snap-frozen clinical specimens are comparable to those in OMM1 and 92.1 cells, suggesting that uveal melanoma cell lines did not acquire Notch ligand expression as an artifact of culture. In loss-of-function studies,

we found that downregulation of Jag2 by shRNA in these lines significantly reduced proliferation, clonogenicity in soft agar, invasion, and migration. We hypothesized that the positive effects of Jag2 on cell proliferation and invasion might be due

FIGURE 7. Downregulation of Jag2 reduces cellular invasion and migration in OMM1 and 92.1 cells. (A, B) Transwell invasion assay in OMM1 (A) and 92.1 (B) cells shows a profound reduction in the invasion capacity when the cells are infected with sh-Jag2 compared with scramble shRNA. Microphotographs in the *right panels* show the invading cells on the lower surface of the protein mixture (Matrigel)-coated filter after 16 hours of incubation. (C) Transwell migration assay performed in 92.1 cells indicates a significant reduction in the number of the cells that had migrated through a gelatin-coated filter after overnight incubation. Microphotographs in the *right panel* show the morphology of the migrated cells. (D) Wound-healing assay shows a reduction in cell migration in 92.1 cells infected with sh-Jag2 compared with scramble shRNA.

at least in part to the activation of ERK1-2, since it has been shown that in breast cancer Notch signaling synergizes with the Ras/MAPK pathway to mediate transformation and poor outcomes.³⁸ Consistent with this concept, phosphorylation levels of Erk1-2 were positively correlated with Jag2 expression and Notch activity in gain and loss of function studies using multiple cell lines.

We also observed that upregulation of Jag2 in Mel285 and Mel290 cells with low endogenous levels of both this ligand and Notch targets was able to increase pathway activity, suggesting that lack of ligand was limiting Notch signaling. Jag2 expression also significantly increased cell growth, invasion, and migration. These tumorigenic effects seem to be mediated at least in part by the activation of canonical Notch signaling, since they were greatly reduced by suppressing the expression of CBF1, the DNA-binding mediator of canonical Notch pathway.

Finally, we found that invading cells express a significantly higher amount of Jag2 mRNA compared with noninvading cells. These data are consistent with a prior expression array analysis performed on both highly and weakly invasive cell subclones derived from a cutaneous melanoma cell line, in which Jag2 was the gene most strongly overexpressed in the highly invasive clone, with RNA levels approximately 15-fold higher than those of the lower invasive cells.³⁹ The fact that Jag2 and Hes1 levels were higher in OMM1 and Mel290 cells, which move more rapidly in transwell assays, suggests that intratumoral heterogeneity with respect to Jag2 levels and Notch activity may also exist in uveal melanoma and affect motility.

Indeed, if we are to understand the role of Notch and its ligands in uveal melanoma biology, it will be important to consider how the pathway interacts with the broader tumor microenvironment. For example, Jag2 expression correlates significantly with angiogenic processes in breast cancer and renal cell carcinoma, and is induced at the transcriptional level under hypoxic conditions.⁴⁰ The oncogene *c-myc* can also modulate Jag2 expression in hypoxia.²⁶ Our culture data indicate that normoxic uveal melanoma cells can express Jag2 and signal to one another in a juxtacrine fashion. However, it will be important to examine Jag2 protein expression in primary tumor material in greater detail to evaluate potential links between the ligand and angiogenesis or vasculogenic mimicry. Expression of Jag2 ligands in the liver or lung might also help uveal melanoma metastases grow at those distant sites.

In summary, our data suggest that Jag2 can promote the growth and dissemination of uveal melanoma cells, but will need to be extended to include analysis of more primary tumor specimens. This Notch ligand has also been suggested to promote tumorigenesis and metastasis in lung adenocarcinoma,⁴¹ breast carcinoma,^{42,43} and multiple myeloma.⁴⁴ Our findings support this growing literature on the importance of Jag2 in tumor growth and metastasis by demonstrating its role in uveal melanoma cells, and suggest that new therapeutic approaches to targeting Jag2 should be investigated in ocular oncology.

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