DNA Damage and Cellular Stress Responses

JAG2 Induction in Hypoxic Tumor Cells Alters Notch Signaling and Enhances Endothelial Cell Tube Formation

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Abstract

Several studies have revealed links between hypoxia and activation of Notch in solid tumors. While most reports have focused on intracellular domain of the Notch1 receptor (icN1) stabilization by direct interaction with HIF proteins, little attention has been given to Notch ligand regulation during hypoxia. Here we show that the Notch ligand JAG2 is transcriptionally activated by hypoxia in a HIF-1α dependent manner. Hypoxic JAG2 induction resulted in elevated Notch activity in tumor cells, as was measured by increased icN1 levels and induction of the Notch target gene HEY1. In primary tumor material, JAG2 expression correlated with vascular development and angiogenesis gene signatures. In line with this, coculture experiments of endothelial cells with hypoxic breast cancer cells displayed a reduction in number of capillary-like tubes formed upon JAG2 siRNA treatment of the breast cancer cells. Together these results suggest that a hypoxic induction of JAG2 in tumor cells mediates a hypoxia-regulated cross-talk between tumor and endothelial cells. Mol Cancer Res; 9(5); 626–36. ©2011 AACR.

Introduction

During growth and expansion of solid tumors, the nutrient and oxygen requirements of the expanding tumor generally surpass that being supplied by the supporting vasculature, leading to environmental oxygen shortage (1). This results in a cellular hypoxic response, primarily orchestrated by the hypoxia-inducible factors (HIF; ref. 2). HIF heterodimers consist of α-subunits (HIF-1α and HIF-2α) and the constitutively stable HIF-1β subunit [ARNT (aryl hydrocarbon receptor nuclear translocator)]. HIF-α and HIF-β subunits heterodimerize within the nucleus where they act as transcription factors, binding to hypoxic response elements (HRE) in HIF target genes such as vascular endothelial growth factor (VEGF; ref. 3, 4). Via the induction of VEGF, among others, the hypoxic response initiates tumor neoangiogenesis. This process is a result of endothelial cell proliferation, migration, and differentiation along the VEGF gradient produced by the tumor (1). In addition to VEGF regulation of vascular development, branching and expansion of the vasculature are under the control of cell–cell activation of Notch signaling (5). Notch signaling is activated by membrane-bound ligand presentation to a Notch receptor on adjacent cells. Notch ligands include the delta-like and jagged ligands (DLL1, DLL3, DLL4, JAG1, and JAG2, respectively). Interaction of these ligands with one of the Notch receptors (NOTCH1-4) results in γ-secretase–mediated cleavage and release of the intracellular domain of the Notch receptor (icN). icN functions as a nuclear transcription cofactor to activate Notch target genes, such as genes of the Hairy/Enhancer of Split (HES) and Hes-related (HEY) families of proteins (6) through interaction with the CBF1/Su(H)/Lag-1 (CSL) [recombination signal binding protein for immunoglobulin kappa J region (RBPJ)] transcription factor. During vascular development, the Notch ligand DLL4 is of particular importance because DLL4-mediated activation of Notch signaling in vasculature results in a process known as lateral inhibition; a process vital for modulating branch formation during angiogenesis (7).

In hypoxic cells cross-talks between HIF and Notch pathways have been reported, primarily resulting in elevated Notch signaling. Several mechanisms have been proposed including direct icN1 stabilization by interaction with HIF proteins (8–18). As of yet, little attention has been given to Notch ligand regulation and the effects this may have on Notch activation under hypoxia. Furthermore, whether hypoxia-induced Notch signaling may influence tumor angiogenesis remains to be investigated. Here we show that hypoxia resulted in elevated expression levels of the Notch ligand JAG2 via transcriptional activation by HIF-1α. This elevation was responsible for a substantial part of the increased Notch activity observed in hypoxic tumor cells. JAG2 expression in primary tumor material was correlated with gene signatures of vascular development and angiogenesis. In addition, coculture of vascular endothelial cells with hypoxic...
tumor cells revealed that JAG2 expression in tumor cells promoted endothelial cell tube formation. Together these results suggest that a hypoxia-regulated cross-talk between tumor and endothelial cells via Notch activation is involved during tumor angiogenesis.

Materials and Methods

Cell culture

**Tumor cells.** MCF7 and T47D cells [ATCC (American Type Culture Collection)] were cultured in RPMI-1640 growth medium, with 10% FBS and 1% penicillin-streptomycin (PEST). T47D cell medium also contained 1% glucose, 1% sodium pyruvate, and 5 μL/mL insulin. Under normoxic conditions cells were cultured in a humidified incubator maintained at 37°C with 5% CO₂ and 95% air (approximately 21% O₂). Under hypoxic conditions the cells were cultured in a hypoxystation H35 cell culture incubator, maintained at 37°C with 1% O₂, 5% CO₂, and 94% N₂.

**Endothelial cells.** MS1 murine endothelial cells were cultured at the same normoxic conditions as described above in EBM-2 medium (Lonza) supplemented with EGM-2 MV SingleQuots (Lonza; hydrocortisone, hEGF, FBS, VEGF, hFGFb, R3-IGF-1, ascorbic acid, and gentamicin/amphotericin-B). The cell culture plates were coated with 1% gelatin.

siRNA transfections for quantitative PCR and protein analysis

Cells were seeded out into 6 well plates (2 wells per si treatment) at a density of 120,000 cells per well. The cells were left to attach overnight. The cell medium (as described above) was replaced and replaced with 2 mL OptiMEM (Invitrogen) without any added FBS or antibiotics. Lipofectamin 2000 (Invitrogen) was used as the transfection agent. Predesigned Silencer Select siRNAs (Ambion) were used to silence desired genes. Transfections were carried out with a Lipofectamin 2000 concentration of 1.5 μL/mL, and siRNA concentrations ranging from 2.5 to 10 nmol/L. Cells were transfected under normoxic conditions (described above) for 6 hours. The transfection medium was then replaced with respective cell media (described above) containing 10% FBS and no antibiotics. Cells were maintained in normoxic culture overnight. Cells were then moved to respective oxygen conditions (21% or 1% O₂) and cultured for 24 hours before harvesting.

siRNA transfections for coculture experiments

Cells were seeded out into as above at a density of 200,000 cells per well. Cells were left to attach overnight. The transfection procedure was carried out as above. Following the 6-hour transfection, the transfection medium was replaced with respective cell media (described above), and the cells were moved to respective oxygen conditions (21% or 1% O₂). The cells were cultured overnight before seeding in the coculture experiments.

Chromatin immunoprecipitation

T47D cells were cultured under hypoxia for 24 hours. Cells were then harvested and immunoprecipitation was carried out using the Diagenode Transcription Factor chromatin immunoprecipitation (ChIP) Kit according to protocol. In brief, the cells were fixed and collected using the scraping method. This was followed by cell lysis in 1× proteosome inhibitor containing lysis buffer. Chromatin shearing was then carried out using a Diagenode Bioruptor sonicator set on high for 7.5 cycles of 30 seconds at 4°C. Immunoprecipitation was carried out using either anti-human IgG antibody (rabbit polyclonal, Abcam) or anti-human HIF-1α antibody (rabbit polyclonal—ChIP grade, Abcam). Precipitated protein–DNA complexes were then de-cross-linked by overnight incubation at 65°C in a thermoshaker. DNA was purified using phenol extraction and used for quantitative real-time PCR (q RT-PCR) using primers specific for a potential HIF binding sites within the JAG2 promoter (Supplementary Table S1B). Primers targeting the known HRE within the VEGF promoter were used as the positive control, and primers targeting a region upstream of the JAG2 promoter were used as a negative control (Supplementary Table S1B). Relative precipitated DNA values were calculated using the Delta-Delta-CT Method.

Quantitative real-time PCR

Cells were lysed and homogenized using the QIAshredder Kit (Qiagen), then loaded onto RNasy spin columns (Qiagen) for total RNA extraction according to the manufacturer’s recommendation. After DNase treatment and extensive washing using Microcon spin columns, cDNA was synthesized using random primers and Multiscribe Reverse Transcriptase enzyme (Applied Biosystems). Generated cDNA was used as template with SYBR Green PCR Master Mix (Applied Biosystems) and expression levels were quantified using the comparative Ct method. Expression levels were normalized using the expression of 3 housekeeping genes (UBC, YWHAZ, and SDHA; ref. 19). Primer sequences are listed in Supplementary Table S1A.

Western blot

For protein extraction, cells were pelleted by centrifugation, then lysed in 10 mmol/L Tris-HCL (pH 7.2), 160 mmol/L NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L EGTA, and 1 mmol/L EDTA [RIPA (radioimmunoprecipitation assay buffer)] supplemented with Complete Protease Inhibitor cocktail (Roche Molecular Biochemicals). The method of Bradford was used to determine protein concentrations. SDS-PAGE separation of proteins in MOPS buffer (Invitrogen) and subsequent blotting onto polyvinylidene difluoride (PVDF) membranes (Millipore) was done using the NuPAGE gels and system (Invitrogen) according to the manufacturer’s instructions. Membrane blocking and antibody dilutions were made in 5% milk diluted in 0.010 mol/L phosphate buffer (pH 7.4), 2.7 mmol/L KCl, 0.140 mol/L NaCl, 0.05% Tween 20.
(PBS-Tween). The following antibodies and antisera were used: actin (MP Biomedicals; diluted 1:2,000), Intracellular Notch-1 (Cell Signaling; diluted 1:500), JAG2 (Abcam; diluted 1:400), and sheep anti-mouse and anti-rabbit secondary antibodies (GE Healthcare; diluted 1:5,000).

Immunohistochemistry and immunofluorescence

For immunohistochemistry, human ductal carcinoma in situ (DCIS) specimens were routinely embedded in paraffin upon fixation. The Envision system and DAKO Technmate 500 were used for detection of protein after incubation of sections with the following primary antibodies: HIF-1α (Upstate; diluted 1:100) and JAG2 (Abcam; diluted 1:250).

For immunofluorescence, cells were grown on cover slips and then fixed in 4% paraformaldehyde. After blocking in 5% normal goat serum and 0.3% Triton X-100 in PBS for 30 minutes, cover slips were incubated for 1 hour with JAG2 antisera (Abcam; diluted 1:400) diluted in PBS, then washed and further incubated for 1 hour with secondary antibody conjugated to Alexa fluor 488 (Molecular Probes). Cover slips were mounted in PVA-DABCO.

Endothelial cell tube formation

Ten thousand MS1 (transformed mouse pancreatic islet endothelial) cells (20; a kind gift from Dr. Kristian Pietras, Karolinska Institutet, Stockholm, Sweden) were mixed with 10⁴ T47D breast cancer cells (treated as indicated), then seeded in 96 well plates on top of layer of growth factor-reduced Matrigel (BD) in EBM-2 medium (Lonza; supplemented with EGM-2 MV SingleQuots (Lonza; hydrocortisone, hEGF, FBS, VEGF, hFGFB, R3-IGF-1, ascorbic acid, and gentamicin/amphoteracin-B). Matrigel was thawed slowly at 4°C overnight, then allowed to polymerize at 37°C for 30 minutes before seeding. Tube formation and length was measured using 3 photographs from each group taken at 8 hour postseeding. For number of tubes formed, each connected tube in each photograph was counted and average values within groups were compared. For measurements of tube length, images were first processed using the ImageJ software and the Tubeness plugin (21) to visualize tubes. Each tube was then identified and total tube length quantified using the Fiji software (http://homepages.inf.ed.ac.uk/s9808248/imagej/tracer/). In both cases, values were normalized to the average value in cells cultured with untransfected T47D cells and statistical significance calculated using a one-way ANOVA followed by Tukey HSD (honestly significant difference) test comparing the control transfected group with JAG2 siRNA groups. For conditioned media experiments, siRNA-treated T47D cells were cultured in EBM2 medium overnight under hypoxia, after which MS1 cells were seeded as above with conditioned media replacing the T47D cells.

Nuclear extracts

Nuclei were extracted after harvesting cells in 40 mmol/L Tris-HCl pH 7.5, 1 mmol/L EDTA, 150 mmol/L NaCl (TEN), then washed in PBS and 25 mmol/L Tris pH 7.5, 50 mmol/L KCl, 2 mmol/L MgCl₂, 1 mmol/L EDTA, 5 mmol/L Dithiothreitol (DTT; Buffer A). Pellets were resuspended in 25 mmol/L Tris pH 7.5, 0.42 mol/L NaCl, 1.5 mmol/L MgCl₂, 0.5 mmol/L EDTA, 1 mmol/L DTT, 25% Sucrose (Buffer NE) and nuclei extracted for 30’. After centrifugation to remove cellular debris, supernatants were used as the nuclear fraction.

Gene expression analysis

JAG2 expression data from Chi and colleagues (22) were analyzed using Oncomine (23). For the JAG2 correlation

Figure 1. Notch signaling is elevated in hypoxia. MCF7 and T47D breast cancer cells were cultured in normoxia (21% oxygen) and hypoxia (1% oxygen). A, Western blot analysis of activated NOTCH1 (sc91) on nuclear extracts. B, QPCR analysis of mRNA expression levels of HEY1. Expression levels are displayed as relative expression levels to those observed in normoxia. Data represent 1 of at least 3 independent experiments run in triplicate. Error bars represent SD. C, relative mRNA levels of the Notch receptors (NOTCH1-4). D, relative mRNA levels of the Notch ligands (DLL1, DLL4, JAG1, JAG2). DLL3 transcript levels are not displayed due to Cq levels being below the detection cutoff point of 35. Average normoxic Cq values obtained from QPCR analysis are displayed. Error bars represent SD. Data represent mean values from 3 independent experiments run in triplicate. * indicates P < 0.05; ** indicates P < 0.01, in a 2-tailed unpaired Student’s t test.
analyses in primary tumor material, 2 gene expression data sets, the first comprising 200 breast tumors (24) and a second comprising 59 clear cell renal carcinoma samples (25) were downloaded from the NCBI (National Center for Biotechnology Information) GEO website (http://www.ncbi.nlm.nih.gov/geo/; accession numbers GSE11121 and GSE14994, respectively). Raw CEL files were RMA normalized in R (http://www.r-project.org) using the Affy package. For both of these data sets, the Pearson correlation was calculated between JAG2 expression and all other reporters present on the array. Genes were then ranked according to their respective JAG2 correlations and gene set enrichment analysis (GSEA; ref. 26) was applied on these ranked lists using the c2.v2.5 curated gene sets collection as supplied by the Molecular Signatures Database (MsigDB; broad.mit.edu/gsea/msigdb). Gene Ontology (GO) analyses were carried out on the 100 reporters showing highest correlation to JAG2 using the Functional Annotation Tool in DAVID (27, 28) and on the complete ranked JAG2 correlation lists using the c5.v2.5 GO gene sets collection in GSEA.

Figure 2. JAG2 protein and mRNA levels are elevated by hypoxia in vivo and in vitro in different tissues/cells. A, immunofluorescence staining of JAG2 protein in T47D cells cultured at normoxia or hypoxia. Inset shows immunohistochemical staining of JAG2 in hypoxic T47D cells. B, immunohistochemical analysis of a breast ductal carcinoma in situ. Antibodies recognizing HIF-1α (left) and JAG2 (right) were used. *, indicates areas magnified in insets. C, JAG2 mRNA expression from public microarray data sets obtained from Oncomine. Expression analysis was carried out on mammary epithelial cells (left) and renal proximal tubule epithelial cells (right) that had been cultured at normoxia, hypoxia or anoxia. D, JAG2 mRNA expression from public microarray data sets on cultured human macrophages (44) and P493-6 Burkitt’s lymphoma cells (45) and QPCR data on cultured human neuroblastoma SK-N-BE(2)C cells at normoxia and hypoxia as indicated. Error bars represent SD. E, gene set enrichment analyses (GSEA) of hypoxia-upregulated gene set carried out on JAG2 correlated ranked gene lists produced from microarray analyses of 200 breast tumors (left; FDR q = 0.009, NES: 1.99) or 59 RCCs (right; FDR q < 0.000, NES: 2.32).
JAG2 expression in hypoxic MCF7 cells treated with siRNA targeting HIF1A and/or HIF2A was obtained from the previously published data set by Elvidge and colleagues (29). This data set was downloaded from the NCBI GEO website with accession number GSE3188.

Results

Notch signaling is enhanced by hypoxia

MCF7 and T47D breast cancer cell lines were cultured at 21% or 1% oxygen (normoxia and hypoxia respectively) for
24 hours. In both cell lines, exposure to hypoxia resulted in increased nuclear icN1 protein levels as indicated by western blot analysis using an antibody specific for the activated icN1 receptor (Fig. 1A). In conjunction with the increased icN1 levels, expression of the known Notch target gene HEY1 (30) was also substantially elevated, as revealed by QPCR (Fig. 1B). These results are in agreement with the notion that hypoxia enhances Notch signaling.

**JAG2 is transcriptionally activated under hypoxia**

To determine potential factors responsible for the increased Notch activation observed under hypoxia, the expression of all Notch receptors and ligands in breast cancer cells were examined using QPCR both at normoxia and hypoxia (Fig. 1C and D). With regards to the Notch receptors, NOTCH1, NOTCH2, and NOTCH4 did not appear to be transcriptionally regulated by hypoxia, but NOTCH3 displayed increased transcript levels (Fig. 1C). It has previously been shown that NOTCH3 can be involved in regulating the activity of icN1 and downstream NOTCH targets (31, 32), and can therefore not be excluded from having an influence on icN1 elevation in hypoxia or effect on overall NOTCH signaling.

The increase in cleaved icN1 levels could also potentially be attributed to changes in Notch ligand expression. Expression analyses revealed that although all Notch ligands, with the exception of DLL3, which fell below detection limits, appeared upregulated under hypoxia, only JAG2 levels were significantly increased (Fig. 1D). Notably, the detection level of the Notch ligand JAG2 was a minimum of 5 PCR cycles lower than that of all other ligands, suggesting highest expression levels of JAG2 amongst the Notch ligands.

**JAG2 is induced by hypoxia in vivo and in vitro in different tissues/cells**

To confirm that the increase in JAG2 observed at the transcriptional level corresponded to an increase at the protein level, JAG2 protein expression was examined by immunofluorescence in T47D cells cultured at normoxia or hypoxia. The hypoxic T47D cells displayed an evident induction of JAG2 protein when compared with those grown at normoxia (Fig. 2A). The detected JAG2 protein appeared to be primarily localized to the cellular membrane (Fig. 2A, inset) as was expected for a functional Notch ligand. In addition, immunohistochemistry was carried out on sections of breast DCIS specimens, which in the comedo form are characterized by necrotic cores with surrounding hypoxic cells (33). JAG2 protein expression was elevated in the hypoxic regions of the tumor surrounding the necrotic cores (Fig. 2B), which was confirmed by detection of HIF-1α protein in the same perinecrotic regions.

To determine if the hypoxic induction of JAG2 was specific to breast cancer or if it could be detected in other cellular systems, expression data from hypoxia-treated cells were examined using the online database Oncomine (23). Here it was found that for both mammary epithelial and renal proximal tubule epithelial cells (22), exposure to hypoxia resulted in increased levels of JAG2 (Fig. 2C). Furthermore, microarray data from macrophage and Burkitt’s lymphoma cells as well as QPCR data on neuroblastoma cells cultured at normoxia and hypoxia showed a consistent upregulation of JAG2 mRNA levels under hypoxic conditions (Fig. 2D).

Moreover, we analysed a data set from a microarray carried out on 200 breast cancers (24) as well as a data set from the breast cancer cell lines T47D and MCF-7. The data set from T47D and MCF-7 showed a significant increase in JAG2 expression in hypoxia compared with normoxia (Fig. 2D). These data are in agreement with the previous studies showing hypoxic induction of JAG2 in mammalian cells.

**Figure 4. JAG2 modulates Notch signaling under hypoxia.** T47D cells were cultured at 21% or 1% oxygen and were treated with control siRNA or siRNA targeting JAG2. A, relative mRNA levels of JAG2 were examined using QPCR and presented as relative expression compared with normoxic levels. Data represent mean values from 3 independent experiments run in triplicate. Error bars represent SD. B, Western blot analysis of JAG2 in control and JAG2 siRNA-treated hypoxic T47D cells. C, Western blot analysis of icN1 on nuclear extracts from untreated, control, and JAG2 siRNA-treated hypoxic T47D cells. D, QPCR-derived relative mRNA levels of HEY1, displayed as relative quantities compared with control-treated normoxic T47D cells. Data represent mean values from 3 independent experiments run in triplicate. Error bars represent SD. *, indicates P < 0.05, in a 2-tailed unpaired Student’s t test.
set consisting of 59 renal cell carcinomas (RCC; ref. 25). For each data set, all genes present on the arrays were ranked according to their correlation to JAG2 expression. GSEA was then applied on the resulting JAG2 correlation ranked gene lists. Consistent in both the breast cancer and RCC arrays, the ranked lists displayed significant enrichment of hypoxia-upregulated gene signatures (Fig. 2E; Supplementary Table S2; FDR $q = 0.009$, NES: 1.99; breast cancer and FDR $q < 0.000$, NES: 2.32; RCC). Together these data show that hypoxia consistently results in increased JAG2 mRNA and protein levels in a variety of cell types, both in vivo and in vitro, and that JAG2 expression in both breast cancer and RCC correlated with transcriptional responses observed under hypoxia, suggesting that JAG2 induction is a general phenomenon in hypoxic cells.

**JAG2 upregulation by hypoxia is HIF dependent**

To determine if the hypoxic induction of JAG2 was HIF dependent, T47D cells were cultured under hypoxia and treated with siRNAs targeting either HIF1A or HIF2A (Fig. 3A and B). JAG2 levels were then monitored using QPCR showing that knockdown of HIF1A, but not HIF2A, diminished JAG2 expression under hypoxia to levels near that detected at normoxia (Fig. 3C). In addition, a publicly available microarray data set on MCF7 cells treated with siRNAs targeting HIF1A and/or HIF2A under hypoxia (29) also displayed a marked reduction in JAG2 levels upon HIF1A but not HIF2A silencing (Fig. 3D). To further confirm HIF-1α involvement in JAG2 induction, chromatin immunoprecipitation experiments were carried out on T47D cells cultured under hypoxia for 24 hours. As a positive control, HIF-1α pull-down on a previously defined VEGF-HRE (34) displayed a 5-fold enrichment compared with the background IgG pull-down (Fig. 3E), and as a negative control, a region upstream of the JAG2 promoter displayed no apparent enrichment (Fig. 3E). After screening a variety of potential HREs located within the promoter region of

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JAG2, HIF-1α pull-down yielded a 12-fold enrichment over the negative control IgG. HIF-1α was found to bind strongest within a sequence ranging from 1,229 to 1,328 base-pairs downstream of the starting ATG of the JAG2 promoter, with a potential HRE at the +1294 position (Fig. 3F). This identified a potential HIF-1α responsive HRE within the JAG2 gene, as well as identified JAG2 as a direct HIF target gene.

**JAG2 contributes to the enhanced Notch activity observed under hypoxia**

Taking into consideration the elevated Notch signaling observed during hypoxia, the role of JAG2 in hypoxic Notch activation was investigated. T47D cells treated with siRNA targeting JAG2 were cultured in normoxia or hypoxia. The JAG2 knockdown was confirmed at both mRNA and protein levels under hypoxia (Figs. 4A and 1B). To examine the effects of diminished JAG2 levels on Notch signaling, icN1 protein levels were again examined by Western blot. The knockdown of JAG2 resulted in a substantial decrease in nuclear icN1 protein levels present under hypoxia (Fig. 4C). In addition, the Notch target gene HEY1 also displayed marked reduction in its hypoxic upregulation upon JAG2 depletion (Fig. 4D). Considering the reduction in both cleaved NOTCH1 levels and hypoxic HEY1 induction, JAG2 elevation strongly contributed to the increase in Notch activity observed under hypoxia.

**JAG2 expression signature in primary tumor material significantly correlates with angiogenic processes**

To further investigate which functional processes are associated with JAG2 expression in primary cancer materials, a GO analysis was carried out on the 100 array reporters showing highest correlation to JAG2 expression. Interestingly, in both breast cancer and RCC arrays, several angiogenic ontology terms were significantly enriched among the JAG2 correlated genes (Table 1), including processes such as blood vessel morphogenesis, blood vessel development, vascular development, and angiogenesis. GO analysis was also carried out on the full JAG2 correlation ranked lists using GSEA, again displaying enrichments for vascular development (Fig. 5A and B; Supplementary Table S2) and angiogenesis (Fig. 5A and B; Supplementary Table S2) in both data sets (FDR q = 0.009, NES: 1.97; breast cancer vascular development; FDR q = 0.023, NES: 1.86; breast cancer angiogenesis; FDR q = 0.04, NES: 1.79; RCC vascular development; FDR q = 0.05, NES: 1.75; RCC angiogenesis).

**Hypoxic JAG2 expression in epithelial tumor cells contributes to endothelial cell tube formation**

Because Notch signaling has a pivotal role in endothelial cell angiogenesis and recent work has displayed interactions between tumor epithelial cells and their surrounding vascular endothelial cells (35), we next investigated whether epithelial JAG2 upregulation under hypoxia might have a

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**Figure 5.** JAG2 correlated gene expression displays enrichment for angiogenic processes. A, GSEAs carried out on a JAG2 correlated ranked gene list produced from a microarray examining gene expression in 200 breast cancers. Significant enrichments were observed for both vascular development (left; FDR q = 0.009, NES: 1.97) and angiogenesis (right; FDR q = 0.023, NES: 1.86) gene signatures. B, GSEAs carried out on a JAG2 correlated ranked gene list produced from a microarray examining gene expression in 70 RCC. Enrichments were observed for both vascular development (left; FDR q = 0.04, NES: 1.79) and angiogenesis (right; FDR q = 0.05, NES: 1.75) gene signatures.
direct role in regulating tumor angiogenesis. To investigate this, an *in vitro* coculture system was employed. T47D cells treated with control or \( \text{JAG2} \) siRNA were cultured under hypoxia for 18 hours before being seeded onto Matrigel in combination with the murine-derived endothelial cells MS1 (20). The tube formation capacity of the endothelial cells was then examined (Fig. 6A). Interestingly, depletion of \( \text{JAG2} \) from the epithelial T47D cell population resulted in a significant reduction in MS1 cell tube formation measured as number of tubes formed as well as total tube length (Fig. 6B and C). This reduction appeared cell-cell contact dependent, as MS1 cells grown with conditioned medium from siRNA-treated hypoxic T47D cells displayed no significant change in tube formation between groups (Fig. 6D).

**Discussion**

It has been previously reported that Notch signaling is elevated during hypoxia (8, 10, 11, 14, 18). Here we show that a substantial part of this elevation can be attributed to increased expression of the Notch ligand \( \text{JAG2} \). The hypoxic induction of \( \text{JAG2} \) was HIF dependent, and we show HIF binding to a potential HRE downstream of the \( \text{JAG2} \) start codon. In human tumors, \( \text{JAG2} \) protein levels were elevated in hypoxic regions of the tumor, and GSEA analysis revealed that \( \text{JAG2} \) expression is correlated with hypoxic gene signatures in primary breast cancer and RCC material.

Until now, reports on Notch activity in hypoxia have largely focused on intracellular interactions between icN and HIFs, resulting in enhanced transcription of Notch downstream targets (8, 10). Even though our results showed that \( \text{JAG2} \) was responsible for a large fraction of the Notch activity under hypoxia, silencing of \( \text{JAG2} \) did not result in complete inhibition of the hypoxic potentiation of Notch signaling. In light of the previous reports, the remaining elevation in Notch activity could be due to direct HIF-icN1 interactions or elevation of other Notch receptors (36).

The development of a hypoxic environment inevitably results in initiation of angiogenesis and recruitment of new blood vessels. It has been shown that hypoxic cells secrete VEGF among other growth factors, creating a VEGF gradient between the hypoxic source and the surrounding vasculature (1). The vasculature is then activated by the secreted factors and extends in the direction of the VEGF gradient (37). The mechanism behind the extension/formation of the new vasculature has been a recent subject of intense interest and has led to development of VEGF inhibitors for antiangiogenic tumor therapies. Recent studies have also unveiled the pivotal importance of Notch signaling in the process of angiogenesis, with specific focus on 2 Notch ligands \( \text{DLL4} \) and \( \text{JAG1} \) (5, 38–42). It is
suggested that branching of new vasculature is a result of modulation of Notch signaling within the endothelial cells. Activation of endothelial cells via secreted VEGF stimulation results in upregulation of DLL4, which in turn activates Notch in adjacent cells in a ligand-dependent manner (7). The VEGF stimulated DLL4-high endothelial cell, termed the "tip-cell," directs the branching along the VEGF gradient. Meanwhile, the adjacent Notch-activated cells, termed the "stalk-cells," become less responsive to VEGF via downregulation of VEGF-receptors, and instead take on the proliferative role driving new vascular formation (7). In addition, stalk-cells also upregulate the Notch ligand JAG1 that, in contrast to the Notch activating role of DLL4, results in inhibition of Notch-activation back on the tip-cell (42). In total, this results in a mechanism in which tip-cells with low Notch-activity drive the branching of new vasculature, whereas stalk-cells with high Notch-activity drive the lengthening of the vessels. This mechanism is supported by multiple studies in which inhibition of Notch signaling during vascular development results in a hyper-branching phenotype (38–41, 43).

Considering the known involvement on Notch in the process of angiogenesis, we speculated that hypoxic induction of JAG2 and its influence on Notch signaling could represent a novel mechanism for tumor-induced vasculogenesis. Upon conducting a GO analysis on primary breast and RCC tumors, highly significant correlations between JAG2 expression and genes involved in angiogenic processes were detected. This was further confirmed by performing a GSEA on complete JAG2 correlated ranked lists, again displaying significant enrichment for vascular development and angiogenesis gene signatures. In addition, coculture experiments using hypoxic breast cancer epithelial cells and murine endothelial cells displayed significantly reduced endothelial tube formation as was measured by both number of branches and length of tubes. Interestingly, it has previously been shown in head and neck squamous cell carcinoma that growth factor–induced JAG1 expression on tumor cells resulted in increased tumor vascularization (35). It was hypothesized that this increase in angiogenic processes was a result of an increase in endothelial Notch-activity due to JAG1 stimulation. However, in light of recent work displaying an inhibitory role of JAG1 on Notch-activity in endothelial cells (42), it seems probable that the increased vascular branching could be attributed to Notch-inhibition as opposed to activation. In line with this model, our coculture experiments also suggest vascular branching due to alterations to endothelial Notch activation via JAG2 stimulation, which according to what is known today, is likely to be a Notch-inhibiting effect. Our findings bring to light the possibility of local tumor cell–endothelial cell activating cues functioning in a juxtacrine manner that would contribute to tumor endothelial cell angiogenesis in addition to the paracrine stimulation via VEGF.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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