Angiocrine Factors Deployed by Tumor Vascular Niche Induce B Cell Lymphoma Invasiveness and Chemoresistance

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SUMMARY

Tumor endothelial cells (ECs) promote cancer progression in ways beyond their role as conduits supporting metabolism. However, it is unknown how vascular niche-derived paracrine factors, defined as angiocrine factors, provoke tumor aggressiveness. Here, we show that FGF4 produced by B cell lymphoma cells (LCs) through activating FGFR1 upregulates the Notch ligand Jagged1 (Jag1) on neighboring ECs. In turn, upregulation of Jag1 on ECs reciprocally induces Notch2-Hey1 in LCs. This crosstalk enforces aggressive CD44+IGF1R+CSF1R+ LC phenotypes, including extranodal invasion and chemoresistance. Inducible EC-selective deletion of Fgfr1 or Jag1 in the Eμ-Myc lymphoma model or impairing Notch2 signaling in mouse and human LCs diminished lymphoma aggressiveness and prolonged mouse survival. Thus, targeting the angiocrine FGF4-FGFR1/Jag1-Notch2 loop inhibits LC aggressiveness and enhances chemosensitivity.

INTRODUCTION

Vascular endothelial cells (ECs) are a specialized component of the tumor microenvironment that can orchestrate tumor growth and invasion (Beck et al., 2011; Bersch and Hanahan, 2008; Butler et al., 2010a; Calabrese et al., 2007; Carmeliet and Jain, 2011; Charles et al., 2010; Ghajar et al., 2013; Lu et al., 2013; Weis and Cheresh, 2011). During regeneration, tissue-specific ECs provide instructive paracrine cues, known as angiocrine growth factors, that trigger proliferation of repopulating progenitor cells (Brantley-Sieders et al., 2011; Butler et al., 2010a, 2010b, 2012; Ding et al., 2014, 2010, 2011, 2012; Potente et al., 2011; Red-Horse et al., 2007). However, the mechanism by which EC-derived angiocrine factors influence tumor behaviors is unknown (Gilbert and Hemann, 2010; Leite de Oliveira et al., 2012; Nakasone et al., 2012; Schmitt et al., 2000).

Notch signaling is a pivotal modulator of lymphomagenesis (Aster et al., 2008; Espinosa et al., 2010; Liu et al., 2010; Lobry et al., 2013), enhancing Myc activity and upregulating receptors such as insulin growth factor-1 receptor (IGF1R) (Medyouf et al., 2011; Weng et al., 2006). The Jagged (Jag) and Delta-like (Dll) families of Notch ligands induce Notch signaling (Gridley, 2010; Siekmann and Lawson, 2007). Both Jag1 and Dll4 are preferentially expressed by ECs during tumor progression but have

Significance

Blood vessels within lymphomas are not just passive conduits delivering nutrients but contain specialized ECs that constitute a maladapted niche actively instigating aggressiveness during tumor progression. Here, we show that tumor ECs supply angiocrine factors to promote chemotherapy resistance and extranodal invasiveness of CD44+IGF1R+CSF1R+ LCs. LCs produce FGF4 to induce expression of Notch ligand Jag1 in ECs. In turn, EC-derived angiocrine Jag1 activates Notch2 in LCs to promote tumor invasiveness and chemoresistance. Interfering with the FGF4/Jag1 cross-talk between LCs and ECs decreases tumor progression and promotes sensitivity to chemotherapy, thereby increasing survival of tumor-bearing mice. Targeting protumorigenic angiocrine factors supplied by lymphoma blood vessels promises efficacious approaches to block tumorigenesis and restore chemotherapy sensitivity without compromising tumor blood perfusion.
distinct roles in neoplastic tissue (Rehman and Wang, 2006; Sethi et al., 2011; Vilimas et al., 2007). Dll4 is expressed by sprouting ECs and appears to regulate EC expansion (proliferative angiogenesis), whereas juxtacrine activation of Notch receptors on tumor cells appears to be mediated by EC-derived Jag1 (inductive angiogenesis) (Lu et al., 2013; Sonoshita et al., 2011; Noguera-Troise et al., 2006; Ridgway et al., 2006). However, mechanisms controlling expression of these Notch ligands in tumor ECs are undefined (Benedito et al., 2009; Corada et al., 2010; High et al., 2008; Hoey et al., 2009; Hofmann et al., 2010; Tung et al., 2012). Moreover, the paucity of EC-specific mouse genetic models has handicapped elucidation of the EC-derived angiocrine signals regulating the fate and behavior of tumors.

Malignant lymphoma cells (LCs) are composed of heterogeneous cell subpopulations, with a subset of LCs possessing more aggressive features (Dierks et al., 2007; Hoey et al., 2009; Kelly et al., 2007). Although chemotherapy eliminates the majority of proliferating LCs, a subpopulation of aggressive LCs manifests resistance, ultimately leading to lymphoma relapse. Because the surrounding microenvironment can support tumor cells (Hanahan and Coussens, 2012; Lane et al., 2009; Memarzadeh et al., 2007; Rakhra et al., 2010; Reimann et al., 2010; Scadden, 2010; Scadden et al., 2012; Trimboli et al., 2009; Zhang et al., 2012), we reasoned that elucidating the microenvironmental signals (i.e., tumor vascular niche) influencing aggressive LCs, such as lymphoma initiating cells (LICs), could provide effective lymphoma treatment strategies.

RESULTS

ECs Support Expansion of LCs with Aggressive Features
To identify the crosstalk between ECs and LCs without the confounding influence of supplementation with exogenous serum and angiogenic growth factors, we devised a serum-containing medium (LCSerum), in serum- and growth factor-free medium (LCSerum), or in serum- and growth factor-free medium with cocultured ECs (LCEC). We found that serum-free coculture of LCs with ECs supported greater LC proliferation than serum alone (Figures 1A and 1B; Figures S1A and S1B available online). Subcutaneous coinjection of LCs with ECs into immunodeficient NOD-SCID-IL2Rγ null (NSG) mice significantly enhanced tumor growth, compared with LCs injected alone (Figure S1C). The growth rate of LCEC in wild-type (WT) C57/B6 mice was significantly higher than LCSerum early after subcutaneous injection (Figures 1D and S1E). We then assessed the serial methylcellulose colony formation (MCF) capacity of expanded LCs. LCEC had 5-fold greater MCF potential than did LCSerum (Figures 1C and 1D). Limiting dilution transplantation into NSG mice showed that LCEC cells contained more lethal LCs than did LCSerum cells (Figure 1E). Thus, ECs establish an inductive vascular niche that enforces outgrowth of aggressive LCs.

We then evaluated whether ECs could confer chemotherapy resistance to LCs by treating LCEC and LCSerum with doxorubicin. Indeed, LCEC were less sensitive to doxorubicin than LCSerum in vitro (Figures 1F and 1G). Doxorubicin prolonged survival of NSG mice transplanted with LCSerum but not mice transplanted with LCEC (Figure 1H). Thus, coculture with ECs promoted a chemotherapy-resistant phenotype in the B220+CD19+My+c + B cell LCs.

To investigate the mechanism underlying the aggressiveness of LCEC, we profiled the transcriptome of LCEC. Coculture with ECs induced transcripts characteristic of LICs, including CD44, IGF1R, and CSF1R (Hanahan and Weinberg, 2011; Medyouf et al., 2011) (Figure 1I; Table S1). Flow cytometry analysis and quantitative PCR (qPCR) confirmed that EC coculture-mediated upregulation of corresponding proteins was due to generation of a CD44+IGF1R+CSF1R+ LC subset (Figures 1J, S1F, and S1G). To assess the functional activity of the CD44+IGF1R+CSF1R+ subset, we obtained clonally derived LCs by serial dilution in coculture with ECs (Figure 1K) and compared their activity to CD44+IGF1R+CSF1R−LCs. The CD44+IGF1R+CSF1R+ LCs yielded more serial methylcellulose colonies, caused higher lethality in limiting dilution transplantation, and were less sensitive to doxorubicin than CD44+IGF1R+CSF1R−LCs (Figures 1L–1O and S1H–S1J). Thus, EC coculture enabled outgrowth of more aggressive and chemoresistant CD44+IGF1R+CSF1R+ LCs.

ECs Support LCs via Jag1-Dependent Juxtacrine Activation of Notch2 Pathway
We next investigated the mechanism by which ECs stimulate aggressiveness in LCs. Transcription profiling showed upregulation of the Notch downstream transcriptional effector Hey1 in LCEC (Figure 1I). qPCR confirmed specific upregulation of Hey1 in LCEC (Figure 2A) and in the aggressive CD44+IGF1R+CSF1R+ LC subset (Figures S2A–S2C). To test whether Hey1 upregulation confers aggressive LC features, we studied how loss and gain of function of Notch1, Notch2, and Hey1 in LCs altered their expansion (Figures 2B and S2D–S2F). Genetic silencing using small hairpin RNA (shRNA) to Notch2 (shNotch2) or Hey1 (shHey1), but not Notch1 (shNotch1), abrogated EC-driven expansion of LCs. In contrast, Hey1 overexpression recapitulated the effect of EC coculture (Figures 2C, 2D, S2G, and S2H). Notch pathway inhibition using the γ-secretase inhibitor compound E similarly abrogated LC growth after coculture with ECs. Immunoblot and immunostaining for Notch intra-cellular domains and chromatin immunoprecipitation (ChIP) of...
Figure 1. Expansion of Myc+ LCs with Aggressive LIC Features after Coculture with ECs in Serum and Cytokine-free Conditions

(A) Representative images of Em-Myc mouse LCs cultured with EC. Scale bar, 200 μm.

(B) Quantification of LC number of Em-Myc mouse LCs cultured in the absence of ECs (LC), with EC (LC EC), or with serum supplementation (LC Serum). *p < 0.02; n = 5. All data are presented as means ± SEM throughout.

(C and D) Representative image (C) and the colony number (D) of colony-forming capacity of LCs after serial passage. Five clones were passed every step. *p < 0.02. Scale bar, 500 μm.

(E) Survival curve of NSG mice i.p. transplanted with the indicated numbers of LCs; n = 6–8.

(F and G) Quantification (F) and representative image (G) of proliferation of LCs after treatment of indicated concentrations of doxorubicin; n = 5. Scale bar, 1,000 μm.

(H) Survival of NSG mice inoculated with 1 × 10^5 indicated LCs and treated or not treated with doxorubicin (chemo) as indicated; n = 6–8.

(I) Heat map presenting the expression level of indicated transcripts in LC EC and LC Serum.

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RBPJ showed selective activation of Notch2, but not Notch1, in LCs (Figures 2E, 2G, S2I, and S2J). Therefore, coculture with ECs selectively activates Notch2 in LCs, resulting in Hey1-dependent expansion of LCs.

Next, we tested how disruption of Notch signaling affected in vivo hepatic tumor seeding following intrasplenic injection (Ding et al., 2010) (Figure 2H). Knockdown of Notch2 or Hey1 or administration of compound E significantly reduced hepatic tumor load, but Notch1 knockdown had little effect (Figures 2I, 2-K, S2K, and S2L). Thus, juxtaparan activation of Notch2-Hey1 by ECs promotes extranodal invasion, a feature of aggressive lymphomas.

Although ECs express several Notch ligands, Jag1 was primarily upregulated by ECs after coculture with LCs (Figure 2L). This finding implicates EC Jag1 as the ligand activating Notch2 in LCs because LCs express negligible Jag1 (Figure S2M). To test this, we knocked down Jag1 in ECs (ECshJag1) and cocultured these feeder cells with Myc+ LCs expressing a Notch reporter (RBPJ-driven GFP). ECshJag1 were less effective than control ECs transduced with scrambled shRNA (ECshScr) in supporting serum- and growth factor-free expansion of LCs and in inducing Hey1 upregulation and Notch activation (Figures 2M–2P). Notably, inhibition of the Notch2-Hey1 pathway in CD44+ IGF1R+ LCs after coculture with ECs had little effect on the aggressive feature (Figures S2N and S2O). Thus, propagation of aggressive LICs in vitro is driven by EC Jag1 expression.

**EC-Specific Upregulation of Jag1 in Human Lymphoma Tissue Accompanies Propagation of Perivascularly Localized Aggressive LCs**

To investigate whether human LCs upregulate Jag1 in ECs, we stained primary human Burkitt’s lymphoma for Jag1 and Hey1. Jag1 was specifically expressed by tumor ECs but not by LCs in all tested lymphomas (Figures 3A and 3B; Figure S3A and Table S2). Notably, Hey1 was preferentially expressed in LCs adjacent to Jag1+ tumor ECs (Figures 3A and 3C). We next investigated whether ECs foster propagation of human Burkitt’s LCs that harbor a translocated MYC gene under the control of immunoglobulin heavy chain gene regulatory elements, as modeled by the Eμ-Mycm mouse. Coculture of CD19+ human Burkitt’s LCs with ECs triggered Notch2-mediated signaling in LCs and outgrowth of the CD44+ IGF1R+ subpopulation (Figures 3D–3F and S3B–S3F). Expansion of human lymphoma colony-forming cells and hepatic lymphoma load was also promoted by coculture with ECs, compared with serum culture (Figures 3G–3J). Disruption of the Notch2-Hey1 pathway during coculture by knockdown of Jag1 in ECs (ECshJag1) or Notch2 in LCs (LCshNotch2) or use of compound E abrogated EC-dependent expansion, colony formation, and extranodal invasiveness of human LCs (Figures 3G–3N and S3G–S3J). Notably, after intrasplenic injection into NSG mice, human LCs induced Jag1 expression in the ECs surrounding the lymphoma nodules but not tumor-free hepatic regions (Figure 3O). Thus, expansion of invasive human Burkitt’s LCs is also driven by instructive angiocrine factors supplied by the vascular niche.

**LCs Induce FGFR1 Signaling in ECs to Prime a Jag1+ Vascular Niche that Reciprocally Reinforces Lymphoma Propagation and Chemoresistance**

We have found that during organ regeneration, activation of VEGF-A/VEGFR2 and FGF/FGFR receptor 1 (FGFR1) signaling in ECs induces expression of angiocrine factors (Ding et al., 2010, 2011, 2014). Hence, we tested whether LCs might co-opt these mechanisms to upregulate Jag1 in tumor ECs and form a malignant vascular niche. Both microarray expression and qPCR analysis showed that coculture with ECs upregulated FGFR4 in mouse and human LCs (Figure 4A). Whereas normal human lymph nodes have negligible FGFR4, we found significant expression of FGFR4 protein in human Burkitt’s lymphoma tissue and preferential activation of FGFR1 in the lymphoma-associated ECs (Figure S4A). To examine whether FGFR1-mediated signaling in ECs was necessary for Jag1 induction, we exposed control ECs and FGFR1-deficient ECs to serum-free or conditioned media (CM) derived from LCs. CM derived from mouse LCs activated Jag1 expression in ECs in a FGFR1-dependent manner (Figures 4B and 4C). Notably, during EC-LC coculture, shRNA knockdown of FGFR1 in ECs or of FGFR4 in LCs blocked Jag1 induction and FGFR1 signaling in ECs (Figures 4D and S4B–S4D). Importantly, after intrasplenic injection, FGFR4-deficient human LCs failed to upregulate Jag1 in the ECs of liver lymphoma nodules of recipient mice (Figure 4E). Therefore, LCs supply FGFR4 to activate FGFR1 on ECs to reinforce Jag1-mediated vascular niche function, driving Notch2-dependent expansion of aggressive LCs.

To determine whether this “feed-forward” loop drives lymphoma tumorigenesis in vivo, we conditionally deleted Fgfr1 specifically in adult ECs by crossbreeding VE-cadherin-CreERT2 mice with Fgfr1flxPflxP mice (Figure 4F) and using tamoxifen to delete Fgfr1 specifically in ECs (Fgfr1ΔECΔiECΔiECΔ/ΔECΔiECΔiECΔiECΔiECΔiECΔiECΔ) (Figures S4E and S4F). To control for Cre-mediated toxicity, we used EC-specific haplodeficient Fgfr1ΔECΔiECΔiECΔiECΔiECΔiECΔiECΔiECΔ mice as control mice. We used a murine B6R2 lymphoma transplantation model to examine the lymphoma growth. Fgfr1ΔECΔiECΔiECΔiECΔ mice, but not Fgfr1ΔECΔiECΔiECΔiECΔiECΔiECΔiECΔiECΔ mice, were inhospitable to subcutaneously and intrasplenically injected B6R2 LCs, resulting in reduced tumor growth and hepatic colonization (Figures 4G–4K). Therefore, activation of Fgfr1 in ECs is required for LCs to establish a protumorigenic vascular niche.

We next crossed Eμ-Myc mice with Fgfr1ΔECΔiECΔiECΔiECΔiECΔiECΔiECΔiECΔ and control mice (Figure 5A) to assess the role of Fgfr1 in establishing a
Figure 2. Angiocrine Effects of Endothelial Jag1 on Notch2 Activation and Propagation of Myc+ Mouse LCs

(A) Expression level of Notch pathway effector Hey1, Hes1, and Hey2 in LCs. (B) Approaches to define Notch pathway activation in mouse LCs. Notch1, Notch2, and Hey1 in LCs were silenced by shRNA (shNotch1, shNotch2, and shHey1), and Notch pathway was blocked by compound E. Cell expansion, Notch activation, colony formation, and hepatic invasiveness were then compared. (C and D) Quantification (C) and representative image (D) of expansion of LCs cocultured with ECs (LC+EC) or cultured in serum-free medium (LC). Srb, scrambled shRNA; OE Hey1, overexpression of Hey1; Comp E, compound E; n = 4. Scale bar, 25 μm. (E and F) Notch1 and Notch2 intracellular domains (ICDs) were detected in LCs by immunoblot (E), and Notch2 ICD in LCs was examined by immunostaining (F). White arrowheads indicate nuclear Notch2 ICD. Scale bar, 10 μm. (G) ChIP analysis of RBPJ activity in LCs after Notch inhibition. shN1 and shN2 denote shRNA against Notch1 and Notch2, respectively; n = 4.
malignant vascular niche. Jag1 was preferentially expressed by VE-cadherin+ ECs within the lymphoma of control (Myc\(^+\) Fgfr1\(^{1\Delta ECG/1\Delta ECG}\)) mice, but not by ECs of Myc\(^+\) Fgfr1\(^{1\Delta ECG/1\Delta ECG}\) lymphomas (Figure 5B). Complementary expression of Hey1 was seen in the perivascular LCs of control but not Myc\(^+\) Fgfr1\(^{1\Delta ECG/1\Delta ECG}\) mice. As a result, Myc\(^+\) Fgfr1\(^{1\Delta ECG/1\Delta ECG}\) survived longer than Myc\(^-\) Fgfr1\(^{1\Delta ECG/1\Delta ECG}\), controls, owing to reduced tumor cell proliferation (Figures 5C–5I).

Because ECs promoted the expansion of chemoresistant LCs, we investigated whether EC-specific deletion of Fgfr1 sensitized Eμ-Myc lymphomas to doxorubicin treatment. Survival of Myc\(^+\) Fgfr1\(^{1\Delta ECG/1\Delta ECG}\) mice, but not Fgfr1\(^{1\Delta ECG/1\Delta ECG}\) mice, was prolonged by chemotherapy (Figure 5I). LCs treated with doxorubicin upregulated FGF4 expression, enabling a vascular niche that confers chemoresistance to LCs (Figure 5J). Hence, FGF1-mediated signaling deploys Jag1 in tumor ECs, establishing a protumorigenic vascular niche that shelters resident LCs from chemotherapy-induced cytotoxicity.

Endothelial Jag1 in Tumor Capillaries Subverts Indolent LCs to Manifest Aggressive Features

To investigate whether enhanced chemoresistance and invasiveness of LCs upon being cocultured with Jag1\(^+\) ECs were due to selective enrichment of an aggressive LC subclone or bestowing aggressive attributes to indolent LCs by tumorigenic vascular niche, we cocultured clonally derived CD44\(^+\)IGF1R\(^+\) after being co-cultured with Jag1\(^+\) ECs. The majority of VE-cadherin+ ECs within the lymphoma of control (Figure 5B). Complementary expression of Hey1 and fluorescent intensity of RBPJ-driven GFP reporter in LCs (N); n = 5. See also Figure S2.

Deletion of Jag1 in ECs of Eμ-Myc Mice (Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\)) Abolishes de Novo Generation of Aggressive LCs

To assess the lymphoma-promoting role of EC Jag1, we selectively deleted Jag1 in Eμ-Myc mice (Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\)), using Myc\(^-\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) as controls. We then isolated nodal B cell LCs and assayed for their aggressive attributes (Figure 7A). During serial methycellulose culture, LCs from Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice had fewer CD44\(^+\)GF1R\(^+\) LCs and yielded substantially fewer colonies than LCs from control mice (Figures 7B–7D). LCs derived from Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice were less capable in killing the recipient mice and were more sensitive to doxorubicin compared with LCs isolated from Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice (Figures 7E and 7F). LCs from Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice also gave rise to fewer and smaller hepatic tumors after intrasplenic seeding (Figures 7G and 7H). Therefore, the protumorigenic Jag1\(^+\) vascular niche endows aggressive features to LCs, driving lymphomagenesis, extranodal invasiveness, and chemoresistance.

Vascular Niche-Derived Jag1 Confers Notch-Dependent Chemoresistance to Myc\(^+\) LCs

To unravel the role of EC-derived Jag1 in lymphoma pathogenesis and development of chemoresistance, we administered doxorubicin to Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) and control mice (Figures 8A and 8B). Notch activity was tracked in LCs by crossing Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice with transgenic Notch reporter (TNR-GFP) mice expressing GFP upon Notch activation (Butler et al., 2010b). Genetic ablation of Jag1 in ECs reduced tumor load and improved survival of Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice, compared with control Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice (Figure 8B; Figure S6A). Moreover, LCs from Myc\(^-\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice were more sensitive to chemotherapy, manifesting as enhanced survival (64% after 8 months) compared with a median survival of ~150 days in controls. Notch was activated in LCs positioned adjacent to VE-cadherin+ ECs in control mice, but not in Myc\(^-\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice (Figures 8C and 8D), indicating that Jag1 supplied by ECs activate Notch signaling in LCs. The enhanced survival of Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice was associated with greater LC death (Figures 8E and 8F). Notably, when EC Jag1 is intact, a subset of perivascularly localized LCs with activated Notch signaling (reported by TNR-GFP cells) was protected from doxorubicin-induced cell death. Therefore, Jag1-expressing ECs establish a chemoresistant microenvironment for LCs via juxtacrine Notch activation (Figure 8G).

To assess whether aberrant EC Jag1 expression could cause vascular abnormalities that might compromise tumor blood supply, we examined vascular perfusion in Myc\(^+\) Jag1\(^{1\Delta ECG/1\Delta ECG}\) mice by intravenously injecting B4-isolecitin to label patent ECs. The majority of VE-cadherin+ ECs within the lymphoma were recognized by B4-isolecitin. Furthermore, tissue staining using the hypoxia marker pimonidazole showed little hypoxia.
Figure 3. Influence of Angiocrine Jag1 on Expansion and Aggressive Features of Human B Cell LCs

(A–C) Expression of Hey1 and Jag1 in patient Burkitt’s lymphoma with MYC translocation were examined. VE-cadherin (VE-Cad) was stained to identify ECs (A). Quantification of Jag1 expression in lymphoma ECs (B) and Hey1 in perivascular LCs (C) is shown. Scale bars, 100 μm and 20 μm in inset.

(D) CD44 and IGF1R expression on human LCs cultured with ECs (LCEC) or with serum (LCSerum).

(E and F) Immunoblot analysis (E) and immunostaining (F) analysis of Notch ICD in LC cultured with serum or with indicated ECs. Srb, Scrambled. Scale bar, 25 μm.

(G and H) Representative image (G) and quantification (H) of colony formation capacity of human LCSerum and LCs cocultured with ECs transduced with Scrambled (LCEC-srb) or Jag1 shRNA (LCEC-shJag1). n = 4. Scale bar, 100 μm.

(I and J) Representative image of hepatic lymphoma (I) and quantification of tumor colony number burden (J) of human LCEC-srb or LCEC-shJag1 intrasplenically injected into NSG mice. LCSerum were also injected for comparison. Scale bar, 50 μm.

(legend continued on next page)
in the lymphoma mass of Myc+Jag1ΔEC/ΔEC mice (Figures S6B–S6D). Therefore, vascular blood supply and oxygen delivery were not compromised in Myc+Jag1ΔEC/ΔEC mice. Taken together, we employed a variety of mouse/human lymphoma models to demonstrate that angiocrine expression of Jag1 activates lymphoma Notch to promote tumorigenicity without affecting the passive perfusion function of tumor vasculature.

**DISCUSSION**

Tumor-initiating cells are believed to acquire aggressive phenotypes via cell-autonomous mechanisms. Here, we challenge this paradigm and demonstrate that in certain lymphomas, the protumorigenic vascular niche dictates tumor aggressiveness. We show that tumor ECs convert indolent CD44+IGF1R-CSF1R+LCs to more aggressive CD44+IGF1R+CSF1R+LIC-like cells manifesting greater tumorigenicity, extranodal invasion, and chemoresistance. The majority of these aggressive tumor features were dependent upon FGF4-driven Jag1 expression by tumor ECs. Disruption of this juxtacrine/angiocrine loop at any level—FGF4, FGF1R, Jag1, or Notch2—severely diminished the aggressiveness of both human and murine lymphomas. Thus, Ep-Myc-driven oncogenesis is not sufficient to provoke aggressive lymphoma behaviors. The LC phenotype is plastic and is determined by cues emanating from the malignant vascular niche rather than by cell-autonomous signaling pathways alone.

The properties of the malignant vascular niche are co-opted via activation of FGF4/FGFR1 signaling in tumor ECs. This signaling induces host ECs to express Jag1 in proximity to neighboring LCs. Importantly, the FGFR1-Jag1 feed-forward signaling loop promotes LC chemoresistance and is further reinforced by chemotherapy administration. In this way, angiocrine Jag1 functionalizes a chemoresistance niche that activates Notch signaling in perivascular LCs and spares them from chemotherapeutics such as doxorubicin. These results suggest a paradigm of tumor propagation whereby a dynamic neoplasms-driven tumor microenvironment shelters tumor cells from chemotherapy and instructively directs them to grow locally and invade distal organs. Thus, targeting the malignant vascular niche should sensitize LCs to chemotherapy and improve outcomes.

Indeed, EC-specific deletion of Fgfr1 or Jag1 in the Fgrf1ΔEC/ΔEC and Jag1ΔEC/ΔEC mice enhanced chemosensitivity and improved mouse survival by abolishing Notch activation in Myc+ LCs after doxorubicin treatment. Similarly, in human lymphoma specimens, we found Jag1 upregulated in tumor ECs and the Notch pathway activated in perivascular human LCs, as originally observed in the murine lymphoma models. These findings highlight the functional interplay between intercellular and tumor cells that depends on the FGF-Notch paracrine/juxtacrine loop. Upregulation of endothelial Jag1 is central to this loop because it endows both mouse and human LCs with aggressive LC features. Similarly, as-yet unrecognized and distinct angiocrine pathways are likely activated within the microenvironment of other tumor types.

Blockade of Jag1 expressed by tumor ECs reduced lymphoma progression without compromising perfusion of the tumor vasculature. Jag1 appeared dispensable for most homeostatic vascular functions because deletion of endothelial Jag1 in adult mice caused no excess mortality or morbidity. Thus, inhibiting the instructive angiocrine signals from a tumor-primed vascular niche can effectively target aggressive LC features. Anti-angiocrine therapeutics need not interfere with tumor perfusion and therefore should not be compromised by tumor hypoxia and rebound angiogenesis that can lead to paradoxical tumor growth (Ebros et al., 2009; Péaz-Ribes et al., 2009).

Comparative analyses of human and mouse lymphoma tissues suggest that our findings may have clinical relevance. Jag1 is upregulated in ECs in human lymphoma specimens. Jag1 upregulation in ECs endows both mouse and human LCs with aggressive LC features. Whether induced expression of Jag1 and activation of Notch pathway in human tumor ECs may also portend poor prognosis is unknown and can only be determined in double-blind multicenter clinical studies. Based on our data, we speculate that patients harboring tumors with the capacity of inducing functional Notch ligands in tumor ECs may be at higher risk for tumor relapse and chemoresistance and be treated with more aggressive therapeutic protocols.

Taken together, our findings demonstrate that tumor cells prime a maladapted vascular niche that reciprocally confers tumors with aggressive, lethal properties: augmenting tumor growth, fostering chemoresistance, and promoting extranodal invasion. Indeed, function of lymphoma-propagating or -initiating cells depends on the protumorigenic state of the vascular niche and cannot be entirely attributed to cell-autonomous malignant properties of tumor cells. For example, an authentic tumor-initiating cell may fail to engrate host tissues with an inhospitable vascular niche, and assays used to identify tumor-initiating cells need to be modified to account for the activation state of vascular niche. Similarly, differences in host EC functions may underlie the tumor tropisms that select common metastatic sites. This study introduces promising therapeutic approaches to improve clinical outcomes for patients with aggressive lymphomas by ejecting LCs from the protumorigenic vascular niche to limit local tumor growth and extranodal invasion while sensitizing LCs to chemotherapy.

**EXPERIMENTAL PROCEDURES**

**Transgenic Reporter and Gene-Targeted Animals**

Jag1flp/loxp mice were provided by Dr. Thomas Gridley (Jackson Laboratories), and Fgrf1flp/loxp mice were obtained from Dr. Michael Simons (Yale University School of Medicine). Generation of inducible EC-specific Fgfr1 knockout mice was carried out as described (Ding et al., 2010, 2011; Wang et al., 2010). In brief, Fgfr1flp/loxp or Jag1flp/loxp mice were bred with VE-cadherin-CreERT2 (cdh5-PAC-CreERT2 transgenic) mice and treated (K–N) Inhibition of Notch1, Notch2, and Hey1 was performed in human LCs before EC coculture, and EC-dependent expansion (K and L) and hepatic tumor load of (M and N) of LCs were determined; n = 4. Scale bars, 1000 μm in (K) and 50 μm in (M).

(O) Jag1 expression in host ECs within the hepatic lymphoma nodule was assessed 14 days after intrasplenic injection of human LCs into NSG mice. Lymphoma mass in the liver is denoted by a dotted line. Scale bar, 50 μm (20 μm in inset).

See also Figure S3 and Table S2.
Figure 4. Reciprocal Instigatory Interactions between LCs and Cocultured ECs In Vitro and Host ECs In Vivo
(A) Expression of angiogenic factors VEGF-A, SDF1, FGF2, and FGF4 in mouse and human LCs cocultured with ECs (LC + EC) or in serum-containing medium (LC); n = 4.
(B–D) ECs transduced with FGFR1 shRNA were stimulated with LC CM. LCs were transduced with Scrambled (LCsrb) and Fgf4 shRNA (LCshFGF4). Jag1 and FGFR1 protein levels (B) and FGFR1 activation (as determined by phosphorylation of FRS-2) (C) in ECs were examined and quantified (D); n = 4.
(E) After human LCs were transplanted into NSG mice via intrasplenic injection, Jag1 induction in ECs associated with hepatic lymphoma nodule was determined. Lymphoma mass is delineated from normal tissue by a dotted line. Jag1 expression in lymphoma ECs is indicated in the inset. Scale bar, 100 μm and 20 μm in inset.
(F and G) Schematic representation of generating Fgfr1iDEC/DEC and control Fgfr1iDEC/+ mice (F) and quantification of growth of subcutaneously injected B6RV2 mouse LCs in these mice (G).
(H–K) B6RV2 lymphoma in the liver was examined in Fgfr1iDEC/DEC and control mice (H). Histological studies of hepatic tumor load were done with H&E staining (I) and fluorescent microscopy scan (J) of liver lobe 14 days after intrasplenic injection of LCs. Proliferation of LCs was determined by Ki67 staining (K). Scale bar, 50 μm in (I) and (K) and 1 mm in (J).
See also Figure S4.
with tamoxifen to induce EC-specific deletion (Fgfr1ΔEC/ΔEC or Jag1ΔEC/ΔEC), as described in Supplemental Experimental Procedures. Lymphoma genesis was induced by transgenic Em-driven Myc. Myc+Jag1ΔEC/ΔEC mice were crossed with TNR mice in which the activation of the Notch pathway results in GFP expression. All animal experiments were carried out under the guidelines of the National Institutes of Health and approved by institutional animal care and use committee at Weill Cornell Medical College, using age/ weight/genetic background-matched animals.

Human Burkitt’s Lymphoma Tissues/Samples
Burkitt’s lymphoma patient specimens were obtained from Weill Cornell Medical College. The procedure was approved by the institutional review board at Weill Cornell Medical College. Patient-related information is identified in Table S2. Human Burkitt’s LCs without Epstein-Barr-virus infection were purchased from American Type Culture Collection (ATCC).

**In Vitro Modeling of Vascular Niche for LC Coculture**
To maintain EC survival in serum/growth factor-free conditions without confounding effects of supplementation with serum, bovine brain extracts and recombinant angiogenic factors (i.e., VEGF-A, FGF2, epidermal growth factors, PDGFs, and angiopoietins); primary freshly purified ECs, such as human umbilical vein ECs; or adult tissue-specific mouse ECs were transduced with E4ORF1 gene (VeraVec ECs; Angiocrine Bioscience). VeraVec ECs maintain their native vascular and microvascular attributes and produce physiological levels of tissue-specific angiocrine factors (Butler et al., 2010b, 2012; Nolan et al., 2013; Seandel et al., 2008). As such, VeraVec ECs establish a responsive unbiased vascular niche model to unequivocally interrogate the role of angiocrine factors in fostering the homeostasis of tumor cells and LICs. Both Em-Myc mouse LCs and human Burkitt’s LCs (ATCC) harboring the c-MYC translocation were utilized for coculture with VeraVec ECs as described in...
Figure 6. Acquisition of Aggressive LIC-like Features in LCs by Jag1-Expressing Vascular Niche

(A–E) Aggressive traits of CD44+IGF1R+CSF1R+ indolent LC colonies after coculture with ECs was investigated (A). CD44+IGF1R+CSF1R+ LC colonies were cocultured with ECs and tested for colony-forming capacity (B and C), CD44 and IGF1R expression (D), and lethality in NSG mice after injection of 5 x 10^3 indicated LCs (E).

(F) Different LC colonies were transplanted into mice with EC-specific deletion of Jag1 (Jag1^iDEC/iDEC). Jag1^iDEC/+ mice were used as control.

(G and H) Propagation of CD44+IGF1R+CSF1R+ LCs in Jag1^iDEC/iDEC and control mice was determined after subcutaneous (G) and intrasplenic transplantation. Representative H&E staining of liver is shown in (H); n = 4. Scale bar, 50 μm.

(I and J) Acquisition of aggressive LIC features in CD44+IGF1R+CSF1R+ LCs after transplantation to control and Jag1^iDEC/iDEC mice. LCs were isolated at day 28 after subcutaneous injection from enlarging tumor mass and analyzed for CD44, IGF1R, and CSF1R (I) and serial colony formation capacity (J). Each derived clone was injected into three mice.

See also Figure S5.
Supplemental Experimental Procedures. For simplicity, we refer to VeraVec ECs as ECs.

To investigate the angiocrine contribution of vascular niche to aggressiveness of LCs/LICs, Jag1 shRNA or scrambled shRNA (Open Biosystems) was used to knock down Jag1 in ECs. Experimental procedures of shRNA knockdown of Notch pathway (Notch1, Notch2, and Hey1) in LCs and Notch ligand Jag1 in ECs are described in Supplemental Experimental Procedures.

Figure 7. Generation of Invasive and Chemoresistant Triple-Positive LC Subpopulation in Myc+ Mice with Conditional Deletion of Jag1 in ECs: Myc+Jag1ECΔEC

(A) Analysis of aggressive attributes of LCs from Myc+Jag1ECΔEC and control Myc+Jag1ECΔEC mice. FACS, fluorescence-activated cell sorting.

(B) Percentage of CD44+IGF1R+ LC subset in Myc+Jag1ECΔEC and control mice.

(C and D) Colony-forming capacity of LCs. Five clones isolated from Myc+ mice were picked for each passage. Representative images (C) and quantification of colony (D) are shown. Scale bar, 500 μm.

(E) Lethality of LCs from control (left) and Myc+Jag1ECΔEC (right) mice after limiting dilution transplantation into NSG mice.

(F) Survival of NSG mice injected with 10^5 indicated LCs with or without treatment of 50 mg/kg doxorubicin; n = 5–8.

(G and H) Representative image (G) and quantification of tumor colony number (H) of hepatic tumor in NSG mice after injection of LCs. Scale bar, 1mm.
Subcutaneous Inoculation and Hepatic Tumor Seeding Model

To monitor tumor propagation in vivo, $5 \times 10^5$ human LCs or $1 \times 10^5$ murine Em-Myc Ems were injected i.p., or $2 \times 10^6$ LCs were injected subcutaneously into immunodeficient NSG mice and mice with the indicated genetic backgrounds. A liver-seeding model via intrasplenic transplantation of LCs was performed as described (Ding et al., 2010). Briefly, the mice were anesthetized, and $5 \times 10^5$ mCherry-labeled human LCs or $1 \times 10^5$ mouse LCs were injected into the parenchyma of the spleen. Splenectomy was carried out after the injection. The mice were sacrificed 14 days after intrasplenic transplantation, and hepatic tumor load was analyzed by...
hematoxylin and eosin (H&E) staining and immunofluorescence whole scan of liver lobe. γ-secretase inhibitor compound E was utilized to abolish Notch pathway activation in LCs. LCs were incubated with 1 μM compound E. For in vivo Notch inhibition, compound E was i.p. injected to mice at 2 mg/kg. Notch2 activation was also determined as described in Supplemental Experimental Procedures.

Flow Cytometric Analysis of LCs
For flow cytometry analysis, LCs were filtered through a 30 μm strainer, pre-blocked with Fc block (CD16/CD32; BD Biosciences), and then incubated with the primary antibodies CD44, CD19, and B220 (eBiosciences); IGF1R (Abcam); and Notch1 and Notch2 (Biolegend). Primary antibodies were conjugated to Alexa Fluor dyes using antibody labeling kits (Invitrogen) (Abcam); and Notch1 and Notch2 (Biolegend). Secondary antibodies to mouse VE-Cadherin (R&D Systems), Jag1 (Abcam), and Ki67 (Dako) supplemented with 10% normal donkey serum/1% BSA/0.1% Tween 20, followed by incubation with fluorophore-conjugated second antibodies (Jackson ImmunoResearch). Images were captured on AxioVert LSM710 microscope (Zeiss).

Immunofluorescent Staining
For immunofluorescence study, cryopreserved sections were incubated with antibodies to mouse VE-Cadherin (R&D Systems), Jag1 (Abcam), and K67 (Dako) supplemented with 10% normal donkey serum/1% BSA/0.1% Tween 20, followed by incubation with fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch). Images were captured on AxioVert LSM710 microscope (Zeiss).

Statistical Analysis of Data
All data are presented as means ± SEM. Comparisons between different groups were made using Student’s t test and ANOVA. Statistical significance was considered as p < 0.05.

ACCESSION NUMBERS
The microarray data are deposited at Gene Expression Omnibus under accession number GSE46368.

SUPPLEMENTAL INFORMATION
Supplemental information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2014.02.005.

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