Crosstalk between tumor and endothelial cells promotes tumor angiogenesis by MAPK activation of Notch signaling

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Summary

While significant progress has been made in understanding the induction of tumor vasculature by secreted angiogenic factors, little is known regarding contact-dependent signals that promote tumor angiogenesis. Here, we report that the Notch ligand Jagged1 induced by growth factors via mitogen-activating protein kinase (MAPK) in head and neck squamous cell carcinoma (HNSCC) cells triggered Notch activation in neighboring endothelial cells (ECs) and promoted capillary-like sprout formation. Jagged1-expressing HNSCC cells significantly enhanced neovascularization and tumor growth in vivo. Moreover, the level of Jagged1 was significantly correlated with tumor blood vessel content and associated with HNSCC development. Our results elucidate a novel mechanism by which the direct interplay between tumor cells and ECs promotes angiogenesis through MAPK and Notch signaling pathways.

Introduction

Tumor angiogenesis is a complex process in which new blood vessels are formed in response to interactions between tumor cells and endothelial cells (ECs), growth factors, and extracellular matrix components. Tumor vessels promote growth and progression of human solid tumors, including head and neck squamous cell carcinoma (HNSCC). New tumor blood vessels penetrate into cancerous growths, supplying nutrients and oxygen and removing waste products (Jung et al., 2002; Folkman, 2002; Kerbel and Kamen, 2004; Stupack and Cheresh, 2004). A large number of studies have demonstrated that tumor cells secrete angiogenic growth factors to stimulate EC proliferation and to induce angiogenesis. Among them, vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors, and it is overexpressed in many human cancers (Jung et al., 2002). Targeting VEGF for human cancer therapy has shown promise in the treatment of colorectal cancer, demonstrating the potential for cancer therapy based upon blocking angiogenesis (Ferrara et al., 2004). However, targeting VEGF for human cancer therapy has not been successful in a variety of other tumor types, suggesting that other factors or components may also play a critical role in tumor angiogenesis (Jung et al., 2002; Kerbel and Kamen, 2004). The identification of those factors and components may have important implications in human cancer therapy.

Angiogenic growth factors secreted by tumor cells or tumor stromal cells can directly bind to their receptors on ECs and stimulate angiogenesis by promoting endothelial sprouting, branching, differentiation, and survival (Folkman, 2002; Sparmann and Bar-Sagi, 2004). For example, VEGF secreted by tumor cells specifically binds to its receptors, VEGF receptors 1 and 2, in ECs (Ferrara et al., 2004). Several important signaling pathways, including mitogen-activating protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and phosphoinositol 3-kinase (PI3K)/Akt, have been found to be induced by angio-

SIGNIFICANCE

Angiogenesis plays a critical role in human tumor growth and development. It is well known that tumor cells can stimulate angiogenesis by secreting proangiogenic factors such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8). However, targeting these factors in human cancer therapy has not been very effective, suggesting that other factors or components may also play a critical role in tumor angiogenesis. In this study, we show that tumor-associated growth factors stimulate the direct interaction between tumor cells and ECs via MAPK and Notch signaling pathways, thereby promoting tumor angiogenesis and tumor growth. Our findings underscore the importance of the direct interplay between tumor cells and ECs in tumor angiogenesis, providing a target for antiangiogenic therapy.

genic growth factors in ECs (Shaulian and Karin, 2002). Activation of these pathways can promote the migration, proliferation, differentiation, and survival of ECs (Jung et al., 2002). Recently, genetic studies have found that Notch signaling plays a critical role in vascular formation during early embryonic development (Xue et al., 1999; Krebs et al., 2000; Lawson et al., 2002; Krebs et al., 2004; Gale et al., 2004; Duarte et al., 2004). Notch was initially identified as a neurogenic gene in *Drosophila* (Artavanis-Tsakonas et al., 1999). Extensive studies have demonstrated that the Notch signaling pathway is highly conserved and plays a critical role in the specification of cell fate during development. In human, there are four *notch* receptor genes (Notch1 to Notch4) and five ligands, two *jagged* genes (*jagged1* and *jagged2*), and three *delta* genes (*delta-like1*, *delta-like3*, and *delta-like4*). Notch signaling is triggered by the direct interaction of cells expressing Notch receptors with cells that express Notch ligands at their surface. Upon Notch receptor-ligand binding, the Notch intracellular domain (Notch-ICD) is cleaved from the membrane by γ-secretase and other putative Notch processing enzymes. Subsequently, Notch-ICD is translocated to the nucleus, where it binds to RBPJκ [the mammalian homolog of Su (H), also known as CBF1 or CSL] to activate transcription of Notch target genes (Lindsell et al., 1995; Kato et al., 1997; Mumm et al., 2000). Importantly, Notch receptors and its ligands have been found to be expressed in ECs (Villa et al., 2001; Liu et al., 2003; Shawber and Kitajewski, 2004). However, the role of Notch signaling in tumor angiogenesis has not been explored.

Squamous cell carcinoma (SCC) is one of the most common cancers in the lung, skin, oral cavity, and head and neck regions. SCC is a very malignant tumor. For example, the 5 year survival rate for patients with HNSCC is one of the lowest for any major cancer and has not been significantly improved over the past decades (Wong et al., 1996; Patel et al., 2001; Forastiere et al., 2001; Mao et al., 2004). The molecular mechanisms that control the development and progression of HNSCC are not fully understood. It is known that the MAPK signaling pathway has been chronically activated in HNSCC (Patel et al., 2001; Mao et al., 2004). Several growth factors and their receptors, including epidermal growth factor (EGF), transforming growth factor α (TGFα), and their receptor, EGF receptor (EGFR), and hepatocyte growth factor (HGF) and its receptor, c-Met, have also been found to be associated with HNSCC development and progression (Patel et al., 2001; Zeng et al., 2002a). All these growth factors can potently promote SCC cell proliferation by activating the MAPK signaling pathway. Also, the activation of MAPK by these growth factors induced SCC cells to secrete proangiogenic factors to promote tumor angiogenesis (Bancroft et al., 2001). Recently, we have found that HGF potently stimulated the MAPK signaling pathway to promote SCC cell survival (Zeng et al., 2002a; Zeng et al., 2002b). In this study, we identified that MAPK activated by growth factors including HGF initiated a novel signaling crosstalk between SCC cells and ECs that promoted tumor angiogenesis and tumor growth.

Results

Induction of Jagged1 in SCC cells by growth factors through MAPK

To further explore the role of growth factors such as HGF in HNSCC development, initially, we were interested in whether

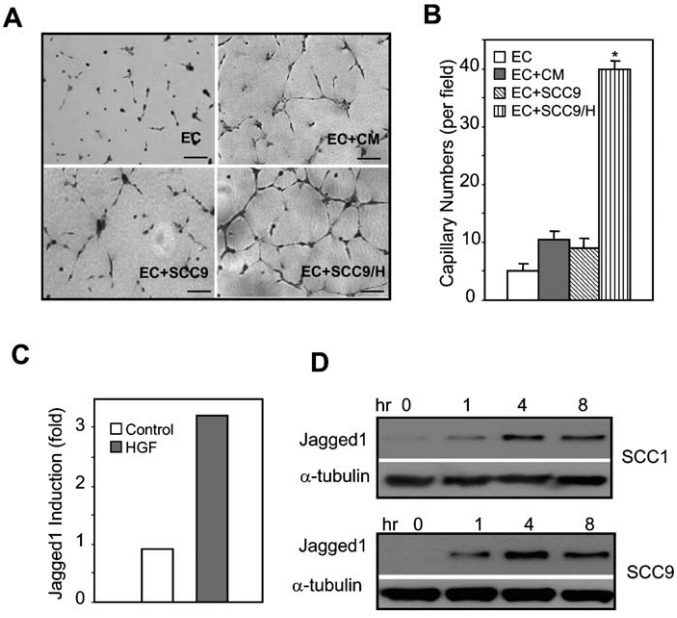

Figure 1. HGF induces Jagged1 expression in SCC cells

A and B: HGF-treated SCC9 cells enhanced endothelial sprout formation. SCC9 cells were untreated or treated with HGF for 4 hr and replaced with the conditioned EGM media (serum-free). After 24 hr, the conditioned media were harvested for EC culture. ECs (0.8×10^4) were plated on the growth factor-reduced Matrigel and incubated with conditioned media. After 24 hr, cells were fixed with methanol and stained with Diff-Quick solution II. For coculture experiments, SCC9 cells were untreated or treated with HGF for 4 hr. Afterwards, ECs (0.8×10^4) and HGF-treated SCC9 cells (SCC9/H; 0.8×10^4) or control SCC9 cells (0.8×10^4) were plated on the Matrigel. After 24 hr, cells were fixed and stained with Diff-Quick solution II. The capillary-like sprouts were counted from five random microscopic fields (×200) and averaged. The assays were performed in duplicate, and the results represent mean values ± SD (error bars) from three independent experiments. Student's t test was performed to determine statistical significance. *$p < 0.01$, EC + SCC9/H versus EC + CM, EC + SCC9, or EC. Scale bar, 50 μm.

C: HGF induced Jagged1 by microarray. Microarray was performed as described previously (Zeng et al., 2002b).

D: HGF induced Jagged1 in SCC cells by Western blot analysis. SCC1 and SCC9 cells were treated with HGF for the indicated time periods. Fifty microgram aliquots of protein extracts were probed with anti-Jagged1 (1:1000) by Western blot analysis. For loading control, the membranes were stripped and reprobed with anti-α-tubulin (1:7500).

HGF stimulated SCC cells to release angiogenic factors to stimulate endothelial differentiation, thereby promoting tumor angiogenesis. Because tumor angiogenesis involves multiple factors, including ECs, extracellular matrix, and tumor cells, a growth factor-reduced Matrigel model system for endothelial differentiation was utilized (Kumar et al., 2004; Liu et al., 2003). In this model system, upon proangiogenic stimulation, ECs proliferate, migrate, and organize into capillary-like sprouts that mimic stages in the development of microvessels in vivo. As shown in Figures 1A and 1B, the conditioned media from HGF-treated SCC9 cells moderately enhanced capillary-like network formation compared with conditioned media from untreated SCC9 cells for 24 hr. In contrast, if we treated SCC9 cells with HGF (SCC9/H) and then used these SCC9/H cells in EC cocultures, a marked induction of networks in the Matrigel was ob-

served compared with untreated SCC9 cells in cocultures for 24 hr. Of note, although some SCC cells may form branching tubules upon HGF treatment for 1–2 weeks, we did not observe that HGF induced SCC9 cells to generate capillary-like sprouts for 24 hr in our model. Moreover, we also confirmed that the sprouts were derived from ECs, but not SCC cells, in cocultures (Figure S1 in the Supplemental Data available with this article online). While other factors might be involved in the enhancement of network formation, our findings led us to examine whether the direct interaction between tumor cells and ECs stimulated a signaling cascade to promote angiogenesis.

Previously, we generated a gene expression profile induced by HGF in SCC cells using microarray (Zeng et al., 2002b). By searching our database, interestingly, although secreted angiogenic factors such as interleukin-8 (IL-8) were upregulated, we found that Jagged1 was strongly induced by HGF (Figure 1C). Jagged1 is a ligand for Notch proteins whose signaling plays a critical role in the specification of cell fate through local cell interactions. Since ECs express Notch receptors (Villa et al., 2001; Shawber and Kitajewski, 2004), the induction of Jagged1 in SCC cells might activate the Notch signaling pathway in ECs. To confirm our microarray data, we examined whether Jagged1 expression was induced by HGF in SCC cells by Western blot analysis. As shown in Figure 1D, upon HGF treatment, the expression of Jagged1 in SCC cells was rapidly induced by HGF in a time-dependent fashion as determined by Western blot analysis.

Recently, we and others found that HGF strongly activated the MAPK and Akt signaling pathways to inhibit apoptosis in SCC cells, both of which have been suggested to play a critical role in the stimulation of gene expression (Zeng et al., 2002a; Zeng et al., 2002b; Birchmeier et al., 2003; Trusolino et al., 2001). Therefore, we determined whether HGF-induced Jagged1 expression was dependent on MAPK and/or Akt activation. As shown in Figure 2, the treatment with the MEK inhibitor U0126, but not the PI3 kinase inhibitor LY294002, significantly suppressed HGF-induced Jagged1 expression as determined by Western blot (Figures 2A and 2B) or Northern blot (Figures 2C and 2D) analysis in SCC9 and SCC14A cells. The specificity of these inhibitors was confirmed in SCC cells by Western blot analysis (Zeng et al., 2002a). Since AP-1 is a major effector of the MAPK signaling pathway (Shaulian and Karin, 2002), we also examined whether the inhibition of AP-1 activity suppressed Jagged1 expression using SCC cells expressing the dominant-negative mutant of c-Jun, TAM-67. Previously, we have demonstrated that TAM-67 was able to inhibit AP activation induced by HGF in SCC cells (Zeng et al., 2002b). To rule out clonal variation, we utilized retrovirus-mediated transduction to generate SCC9 and SCC14A cells that stably expressed TAM-67 (Figures 2E and 2F, top panel). While Jagged1 was induced in control SCC cells following HGF treatment, the induction of Jagged1 was suppressed in SCC cells expressing TAM-67 (Figures 1E and 1F, bottom panel). Consistently, we found that two close AP-1 binding sites at positions –2488 and –2616 were present in the region of the *jagged1* promoter. Our chromatin immunoprecipitation (ChIP) assays found that the AP-1 components c-Jun and c-Fos were recruited to this region following HGF stimulation (Figure S2).

We also directly examined whether the activation of the MAPK signaling pathway by EGF or TGFα could induce Jagged1 expression in SCC cells. Importantly, EGFR has been

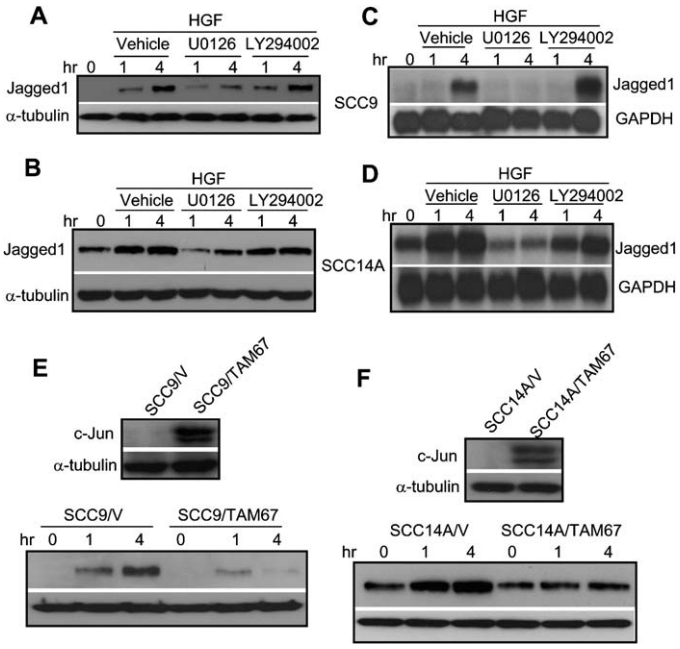

Figure 2. HGF induces Jagged1 expression through MAPK

A and B: MAPK-dependent Jagged1 expression by Western blot analysis. Both SCC9 and SCC14A cells were pretreated with U0126 or LY294002 for 30 min and then treated with HGF for the indicated time periods. Fifty microgram aliquots of protein extracts were probed with anti-Jagged1.

C and D: MAPK-dependent *jagged1* expression by Northern blot analysis. Five microgram aliquots of total RNAs were probed with ^{32}P-labeled human *jagged1* cDNA probe. As an internal control, the membrane was stripped and reprobed with ^{32}P-labeled *glyceraldehydes-3-phosphate dehydrogenase* (*GAPDH*) cDNA probe.

E: The inhibition of AP-1 suppressed Jagged1 expression induced by HGF in SCC9 cells. To establish SCC9 cells stably expressing TAM-67, cells were infected with retroviruses expressing TAM-67 or control vector and selected with G418 (600 μg/ml) for 10 days. The resistant clones were pooled and probed with anti-c-Jun. Both SCC9/V and SCC/TAM-67 cells were treated with HGF (40 ng/ml) for the indicated time periods. Fifty microgram aliquots of protein extracts were probed with anti-Jagged1.

F: The inhibition of AP-1 suppressed Jagged1 expression induced by HGF in SCC14A cells. The experimental procedures were performed as described in **E**.

found to be overexpressed in human HNSCC (Mao et al., 2004). As shown in Figure 3A, the treatment of EGF or TGFα potently induced the activation of ERK in SCC14A cells as well as other SCC cells, suggesting that the EGFR functionally transduced the MAPK signaling cascade in SCC cells. Similarly, the expression of Jagged1 was significantly induced in these SCC cells by EGF or TGFα (Figure 3B). Moreover, the inhibition of MAPK, but not Akt, also suppressed Jagged1 expression in SCC14A cells and other cells (Figure 3C; data not shown). Taken together, the expression of Jagged1 can be induced by common tumor-associated growth factors that activate the MAPK signaling pathway.

Promotion of endothelial capillary-like sprout formation by Jagged1-expressing SCC cells through activating Notch signaling

Next, we examined whether the induction of Jagged1 in SCC cells had a functional role in endothelial differentiation. Since

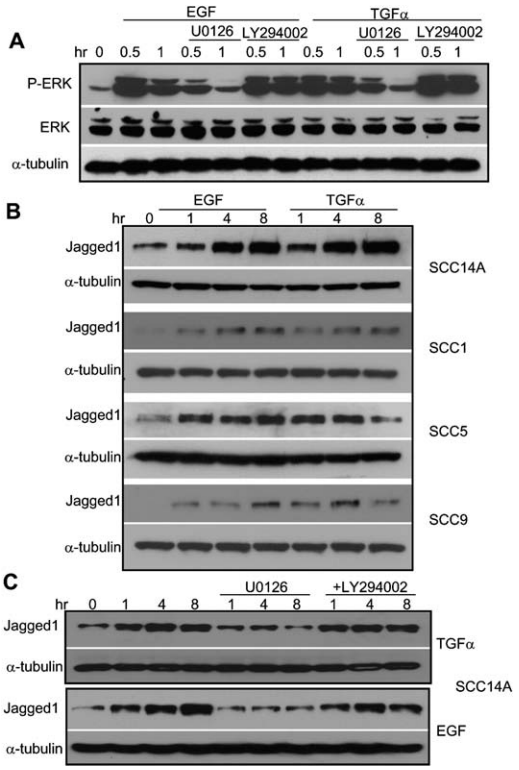

Figure 3. EGF or TGFα induces Jagged1 expression in SCC cells through MAPK

A: EGF or TGFα activated MAPK. SCC14A cells were pretreated with U0126 or LY294002 and then treated with EGF or TGFα for the indicated time periods. Fifty microgram aliquots of protein extracts were probed with anti-phospho-ERK (1:1000). For loading control, the membrane was stripped and reprobed with anti-ERK (1:1000) or anti-α-tubulin.

B: EGF or TGFα induced Jagged1 expression in SCC cells. SCC cells were treated with EGF or TGFα for the indicated time periods. The Jagged1 expression was examined as described in Figure 1.

C: The inhibition of MAPK suppressed Jagged1 expression induced by EGF or TGFα. The experiments were performed as described in Figures 2A and 2B.

HGF, EGF, or TGFα also induced SCC cells to secrete angiogenic growth factors (Mao et al., 2004), it was critical to determine whether the Jagged1-expressing SCC cells alone was capable of inducing endothelial sprout formation by activating Notch signaling in ECs. We utilized retrovirus-mediated transduction to stably express Jagged1 in SCC9 cells in which the level of endogenous Jagged1 was undetectable. Western blot analysis demonstrated that Jagged1-expressing SCC9 cells (SCC9/Jag) and control cells (SCC9/V) were generated (Figure 4A). The coculture of ECs with SCC9/Jag cells, but not with SCC9/V cells, potently activated the Notch-dependent luciferase reporter in ECs (Figure 4B), confirming that Jagged1-expressing SCC9 cells could functionally trigger the Notch signaling pathway in their neighboring cells. To determine whether Jagged1-expressing SCC cells stimulated the capillary-like network formation, ECs were cocultured with SCC9/Jag cells or SCC9/V cells on the Matrigel. As shown in Figures 4C and 4D, SCC9/Jag cells induced 4-fold more endothelial sprouts than SCC-9/V cells. Moreover, these endothelial sprouts induced by SCC9/Jag cells formed a well-connected network.

Since the expression of Jagged1 might activate the Notch signaling pathway in adjacent SCC cells, it was possible that the Notch-inducible genes in SCC cells stimulated endothelial differentiation via a paracrine mechanism. To rule out this possibility, we overexpressed the constitutively activated Notch-ICD in SCC9 cells (Figure 4E). It is well known that, upon ligand binding, the Notch-ICD is cleaved and then moved to the nucleus, where it interacts with RBPJκ to activate gene expression. Previously, there were many studies that have demonstrated that Notch-ICD expression phenocopied Notch signaling (Lindsell et al., 1995; Kato et al., 1997; Mumm et al., 2000). In contrast to SCC9/Jag cells, Notch-ICD-expressing SCC9 cells did not significantly enhance endothelial sprout formation in the Matrigel compared with coculture of ECs with SCC9/V cells (Figures 4C and 4D). The results suggest that the activation of the Notch signaling pathway in ECs, but not in SCC cells, was critical for endothelial sprout and network formation. The luciferase reporter assay confirmed that the overexpression of Notch-ICD stimulated transcription.

Next, we directly determined whether the endothelial sprout formation was directly dependent on the activation of the Notch signaling pathway in ECs. Thus, we utilized a highly specific γ-secretase inhibitor (γ-SI) to inhibit the Notch cleavage and activation (Dale et al., 2003) in the coculture of ECs and SCC9/Jag cells. As shown in Figure 4F, γ-SI strongly suppressed SCC9/Jag cell-induced endothelial network formation. In contrast, we found that γ-SI did not inhibit the proangiogenic factor IL-8-induced endothelial network formation (Figure S3), suggesting that γ-SI may specifically inhibit Notch signaling. The Notch-dependent luciferase reporter assay demonstrated that γ-SI blocked SCC9/Jag cell-mediated Notch transcription in ECs (Figure 4B).

To further validate our results, we also stably expressed the dominant-negative form of Su(H) [(DN-Su(H)] (Artavanis-Tsakonas et al., 1999) in ECs or SCC9/Jag cells by retrovirus-mediated transduction, respectively (Figure 4G). Although Su(H)/RBPJκ-independent activities of Notch are known, Su(H)/RBPJκ is a major effector of the Notch signaling pathway. As shown in Figure 4H, overexpression of DN-Su(H) in SCC9/Jag cells did not have significant effects on endothelial sprout formation in our coculture assay. In sharp contrast, overexpression of DN-Su(H) in ECs totally abolished SCC9/Jag cell-induced network formation. Additionally, we found that the overexpression of Notch-ICD in ECs also promoted the sprout formation in the Matrigel, which could not be inhibited by γ-SI or soluble Jagged1-conditioned media (Figure S4). Taken together, these results suggest that Jagged1-expressing SCC cells were capable of initiating a crosstalk with ECs through activating the Notch signaling pathway, thereby inducing endothelial capillary-like sprout networks.

Endothelial capillary-like network formation induced by endogenous Jagged1-expressing SCC cells

As demonstrated in Figure 3B, SCC14A cells expressed a high basal level of endogenous Jagged1. Thus, we wondered whether the elevated endogenous Jagged1 would promote endothelial differentiation in vitro. As shown in Figures 5A and 5B, like SCC9/Jag cells, coculture of ECs with SCC14A cells strongly promoted endothelial network formation in the Matrigel. To determine whether SCC14A cell-stimulated endothelial sprout formation was dependent on the Notch signaling path-

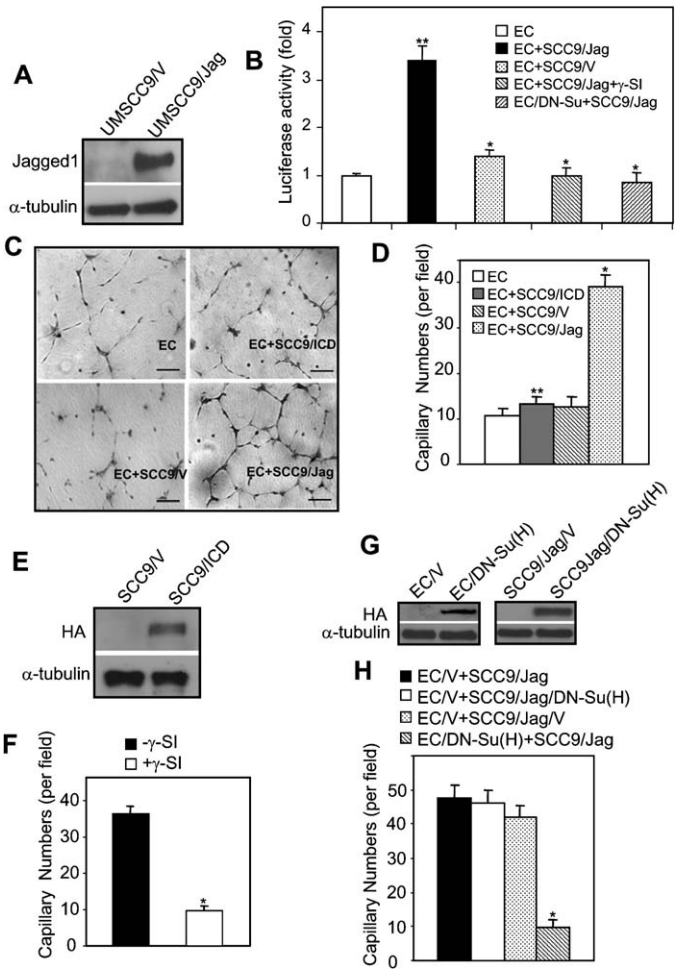

Figure 4. Jagged1-expressing SCC cells promote endothelial capillary-like sprout formation through activating the Notch signaling pathway in ECs

A: SCC9 cells stably expressing Jagged1. SCC9 cells were transduced with retroviruses expressing Jagged1 or control vector. After selection, the stable clones were pooled and probed with anti-Jagged1.

B: Jagged1-expressing SCC9 cells activated Notch-dependent transcription in ECs. ECs were transfected with Notch-dependent luciferase reporter for 24 hr and then cocultured with SCC/Jag cells or control cells (SCC/V) in the absence or presence of γ-SI (50 μM). The luciferase activities were measured with a dual luciferase assay system. In some experiments, ECs were also cotransfected with DN-Su(H). The assay was performed in duplicate, and the results represent mean ± SD (error bars) from three independent experiments. Student's t test was performed to determine statistical significance. **$p < 0.01$, EC + SCC9/Jag versus EC or EC + SCC9/V; *$p < 0.01$, EC + SCC9/Jag versus EC + SCC/Jag + γ-SI or EC/DN-Su + SCC9/Jag.

C and D: Jagged1-expressing SCC cells enhanced capillary-like network formation. ECs were cultured alone or cocultured with SCC9/V, SCC9/Jag, or SCC9/ICD (SCC9 expressing Notch-ICD) for 24 hr. The sprouts were stained and counted as described in Figure 1. Student's t test was performed to determine statistical significance. **$p > 0.05$ (no significant differences), EC + SCC9/ICD versus EC + SCC9/V or EC; *$p < 0.01$, EC + SCC9/Jag versus EC + SCC9/ICD. Scale bar, 50 μm.

E: SCC9 cells expressing Notch ICD. SCC9 cells were stably infected with retroviruses expressing HA-Notch-ICD or control vector.

F: The inhibition of endothelial tube formation by γ-SI. ECs were cocultured with SCC9/Jag cells in the presence or absence of γ-SI (50 μM) for 24 hr. The assay was performed in duplicate, and the results represent mean ± SD (error bars) from three independent experiments. Student's t test was performed to determine statistical significance. *$p < 0.01$, +SI versus −SI.

G: ECs or SCC9 cells stably expressing DN-Su(H). ECs or SCC9 cells were infected with retroviruses expressing HA-DN-Su(H) or control vector. ECs expressing DN-Su(H) [EC/DN-Su(H)] or SCC9/Jag cells expressing DN-SU(H) [SCC9/Jag/DN-SU(H)] were confirmed with anti-HA epitope.

way, we added γ-SI to the coculture. As shown in Figures 5A and 5B, γ-SI totally abolished endothelial tube formation induced by SCC14A. The luciferase assay confirmed that γ-SI inhibited Notch-dependent transcription induced by SCC14A cells (Figure 5C). Also, we utilized soluble Jagged1, which was able to be secreted and to interfere with the interaction between the normal Notch receptor and ligand (Wu et al., 2001). SCC14A cells were stably transduced with retroviruses expressing soluble Jagged1 (S-Jag). As shown in Figure 5D, the S-Jag proteins were detected not only in cell lysates but also in cell culture media, demonstrating that S-Jag was secreted in SCC14A cells. Moreover, S-Jag also inhibited Notch-dependent transcription induced by SCC14A cells (Figure 5C). Compared with SCC14A/V cells, the endothelial network formation was significantly reduced in coculture of ECs with SCC14A/S-Jag cells (Figures 5A and 5B). To further confirm our results, we also determined whether depletion of Jagged1 expression in SCC14A cells by siRNA inhibited sprout formation in our coculture assay. As shown in Figure 5E, Western blot analysis demonstrated that Jagged1 siRNA, but not luciferase siRNA, completely suppressed Jagged1 expression in SCC14A cells. While luciferase siRNA did not interfere with the endothelial sprout formation in our coculture assay, the depletion of Jagged1 expression in SCC14A potently suppressed the sprout formation induced by SCC14A cells (Figure 5F).

Finally, we also determined whether Jagged1 expressed in SCC9 cells was responsible for the capillary-like sprout formation in our coculture assay upon HGF stimulation. As shown in Figure 5G, Western blot analysis confirmed that Jagged1 siRNA, but not control luciferase siRNA, completely inhibited Jagged1 expression in SCC9 cells induced by HGF. Transfection of luciferase siRNA did not affect the sprout formation in our coculture assay upon HGF stimulation. The deletion of Jagged1 by siRNA strongly suppressed capillary-like sprout formation stimulated by HGF-treated SCC9 cells (Figure 5H). Moreover, as shown in Figure 5H, both S-Jag-conditioned media from SCC14A/S-Jag cells and γ-SI also abolished the sprout formation induced by HGF-treated SCC9 cells (Figure 5H). Taken together, these results suggested that the endogenous Jagged1 in SCC cells could initiate the contact-dependent signaling in ECs through the Notch receptor, thereby stimulating endothelial differentiation.

Tumor angiogenesis and tumor growth enhanced by Jagged1-expressing SCC cells in a nude mouse model

To determine whether Jagged1-expressing SCC cells modulated tumor angiogenesis in vivo, we utilized a well-established SCID mouse model of tumor angiogenesis that mimics human tumor angiogenesis in vivo using human ECs. For this model system, both human ECs and SCC cells were mixed with the Matrigel and seeded in porous poly(L-lactic acid) (PLLA) scaffolds. The scaffolds were subcutaneously implanted in the dor-

H: The inhibition of Notch signaling in ECs suppressed sprout formation. EC/DN-SU(H) cells or control cells (EC/V) were cocultured with SCC/Jag/DN-SU(H) cells or control SCC cells (SCC/Jag/V) on the Matrigel as described in Figure 1. The results represent mean ± SD (error bars) from three independent experiments. *$p < 0.01$, EC/DN-Su(H) + SCC9/Jag versus EC/V + SCC9/Jag, EC/V + SCC9/Jag/DN-Su(H), or EC/V + SCC9/Jag/V.

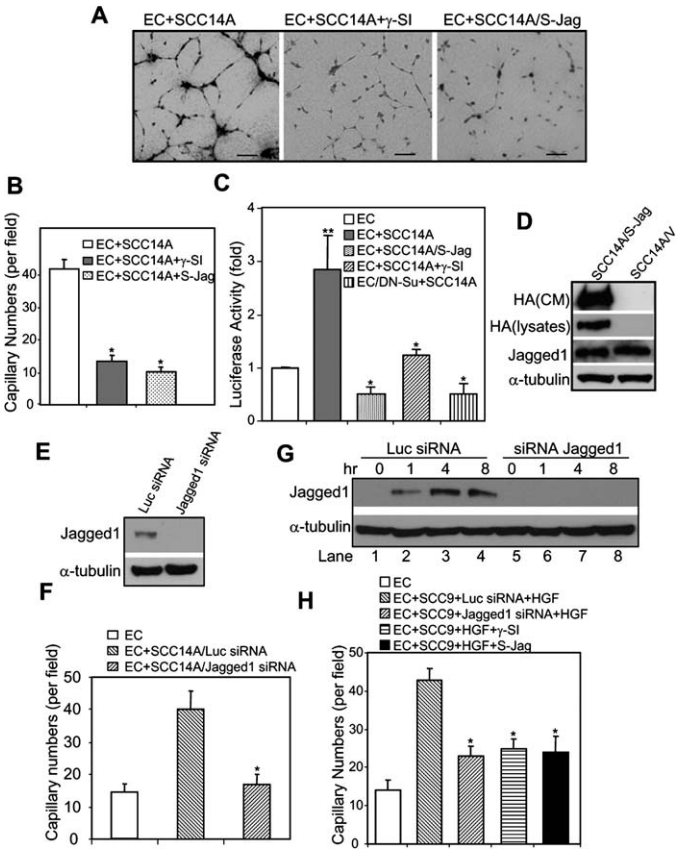

Figure 5. Endogenous Jagged1-expressing SCC cells enhance endothelial capillary-like network formation

A and B: γ-SI or soluble Jagged1 inhibited endothelial sprout formation induced by SCC14A cells. ECs were cocultured with SCC14A cells in the absence or presence of γ-SI or with SCC14A expressing soluble Jagged1. *p < 0.01, EC + SCC14A versus EC + SCC14A + γ-SI or EC + SCC14A/S-Jag. Scale bar, 50 μm.

C: γ-SI or soluble Jagged1 inhibited Notch activation in ECs induced by SCC14A. ECs were transfected with Notch luciferase reporter for 24 hr and then cocultured with SCC14A cells in the absence or presence of γ-SI or SCC14A cells expressing soluble Jagged1 (SCC14A/S-Jag) for 24 hr. The luciferase activities were measured with a dual luciferase system. **p < 0.01, EC + SCC14A versus EC; *p < 0.01, EC + SCC14A versus EC + SCC14A/S-Jag, EC + SCC14A + γ-SI, or EC/DN-Su + SCC14A.

D: SCC14A cells stably expressing soluble Jagged1. SCC14A cells were infected with retroviruses expressing HA-soluble Jagged1 (S-Jag) or control vector. Both supernatants and cell lysates were probed with anti-HA.

E: The depletion of endogenous Jagged1 expression in SCC14A cells by Jagged1 siRNA. SCC14A cells were transfected with Jagged1 siRNA or luciferase (Luc) siRNA (100 μM) for 36 hr, and the expression of Jagged1 proteins was examined by Western blot analysis. For internal controls, the blot was stripped and rechecked with anti-α-tubulin.

F: The inhibition of Jagged1 expression suppressed the proangiogenic effects of SCC14A in coculture. SCC14A cells were transfected with Jagged1 siRNA or Luc siRNA for 36 hr. After treatment, Jagged1- or Luc siRNA-transfected SCC14A cells were cocultured with ECs for 24 hr. *p < 0.01, EC + SCC14A/Luc siRNA versus EC + SCC14A/Jagged1 siRNA.

G: HGF-induced Jagged1 expression was inhibited by Jagged1 siRNA in SCC9 cells. SCC9 cells were transfected with Jagged1 or Luc siRNA (100 μM) for 36 hr and then treated with HGF for 0, 1, 4, and 8 hr. The level of Jagged1 expression was examined by Western blot analysis.

H: The inhibition of Notch signaling suppressed the proangiogenic effects of SCC9 coculture upon HGF stimulation. SCC9 cells were transfected with Jagged1 or Luc siRNA for 36 hr and then treated with HGF for 4 hr. After treatment, Jagged1- or Luc siRNA-transfected SCC9 cells were cocultured with ECs for 24 hr. HGF-treated SCC9 cells were also cocultured with ECs in the presence of γ-SI or soluble Jagged1-conditioned media from SCC14A/S-Jag cells. *p < 0.01, EC + SCC9 + Luc siRNA + HGF versus EC + SCC9 + HGF + Jagged1 siRNA, EC + SCC9 + HGF + γ-SI, or EC + SCC9 + HGF + S-Jag. The results represent mean ± SD (error bars) from three independent experiments. Student's t test was performed to determine statistical significance.

sal region of SCID mice, and tumor angiogenesis was examined by histology and immunostaining (Nor et al., 2001). First, we examined tumor angiogenesis at an early stage (3 weeks). As shown in Figure 6A, the scaffold coimplanted with ECs and SCC9/Jag cells was more vascularized than that with ECs and SCC9/V cells. Along with tumor formation, immunostaining of human factor VIII, a marker for ECs, found that significantly more intratumoral blood vessels were induced in the scaffolds seeded with the combination of ECs with SCC9/Jag cells compared with SCC9/V cells (Figures 6B and 6C). After 6 weeks, scaffolds were retrieved, and the coimplants of ECs with SCC9/Jag cells exhibited more aggressive tumor growth than those with SCC9/V cells, as determined by tumor volume and size (Figures 6D and 6E). Finally, human factor VIII immunostaining also found that there were three times more blood vessels in tumors from the endothelial coimplants with SCC9/Jag cells than in those with SCC9/V cells (Figure 6F).

During in vitro cell culture, we observed that both SCC9/V and SCC9/Jag cells grew at the similar rate. More specifically, the 5-bromodeoxyuridine (BrdU) incorporation assay indicated that the rate of DNA synthesis of both cells in vitro was identical (Figures S5A–S5C). It suggested that the different tumor growth rates from coimplants were unlikely due to the possibility that Jagged1 directly stimulated SCC cell proliferation. To determine whether the enhancement of tumor angiogenesis might affect the proliferation of SCC cells in vivo, tumor-bearing mice were injected with BrdU intraperitoneally, and BrdU uptakes in tumor cells were examined. Unlike in vitro, the immunostaining found that significantly greater numbers of BrdU-positive tumor cells (over 2-fold) were observed in tumors from coimplants of ECs with SCC9/Jag cells compared with those from coimplants of ECs with SCC9/V cells (Figure 6G and Figure S5D), suggesting that the increasing tumor angiogenesis promoted tumor cell proliferation in vivo. Taken together, these results from in vivo studies suggest that Jagged1-expressing tumor cells can stimulate tumor angiogenesis and growth in vivo by interacting with neighboring ECs through the activation of the Notch signaling pathway.

Jagged1 expression in human HNSCC tissues

Although ECs may also express Notch ligands, our results suggest that tumor cells that express Jagged1 may help to direct neovasculature during tumor growth. To explore that the Notch signaling pathway was associated with human tumor angiogenesis, we also examined whether there was corelationship between Jagged1 expression and tumor blood vessel formation in HNSCC tumor tissues by the combination of tissue microarray (TMA) and immunostaining. Previously, the Notch signaling components were found to be overexpressed in human HNSCC by gene profiling (Leethanakul et al., 2000). Thus, we further compared the level of Jagged1 expression in human HNSCC with normal epithelial tissues and dysplasias using the combination of high-density TMA and immunostaining. Repre-

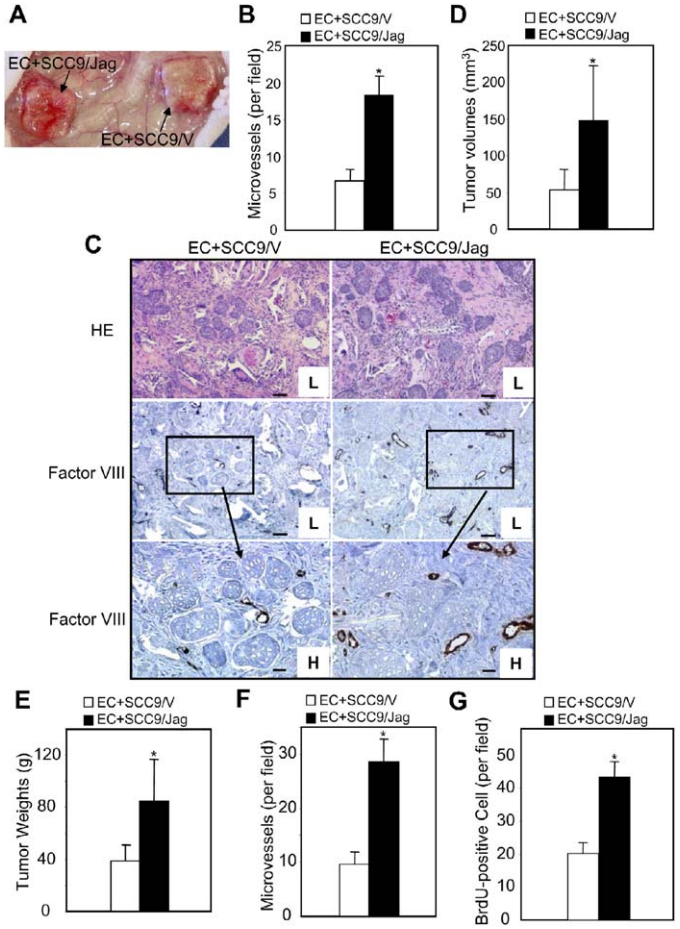

Figure 6. Jagged1-expressing SCC cells promote tumor angiogenesis and tumor growth in a nude mouse model

A: Coimplant of ECs with SCC9/Jag was highly vascularized in vivo. ECs and SCC9/Jag or SCC9/V cells were mixed with the Matrigel, seeded in different scaffolds, and then implanted in SCID mice. After 3 weeks, the implants were harvested and photographed. Left: the coimplant of ECs and SCC9/Jag cells; right: the coimplant of ECs and SCC9/V cells.

B and C: Jagged1-expressing SCC cells enhanced tumor angiogenesis. Coimplants of ECs and SCC9/Jag or SCC9/V cells were sectioned and stained with hematoxylin and eosin (HE) or anti-factor VIII (1:200). The microvessels were counted from five random fields (×200) and averaged. The experiments were performed three independent times (five coimplants per group), and the results represent mean ± SD (error bars) from 15 coimplants per group. Student's t test was performed to determine statistical significance. *$p < 0.01$. L, low magnification, scale bar, 50 μm; H, high magnification, scale bar, 25 μm.

D–F: Jagged1-expressing SCC cells promoted tumor angiogenesis and tumor growth. The experiments were performed as described in **A**, and scaffolds were harvested 6 weeks after implantation. After tumor volumes and weights were measured, specimens were fixed, paraffin-embedded, and sectioned. Serial sections were stained with HE or polyclonal antibodies against human factor VIII. The microvessels were counted from five random fields (×200) and averaged. Two independent experiments were performed (five coimplants per group), and the results represent mean ± SD (error bars) from ten coimplants per group. Statistical significance was determined by the Student's t test. *$p < 0.01$.

G: The promotion of BrdU uptake by SCC cells in tumors derived from the coimplants of endothelial cells and SCC9/Jag cells. The experiments were performed as described in **A**. Mice were injected with BrdU labeling reagents (10 ml/kg) intraperitoneally 2 hr before the scaffolds were retrieved. The sections were incubated with biotinylated anti-BrdU using the Zymed BrdU staining kit. The BrdU-positive cells were counted from five random fields (×200) and averaged. Two independent experiments were performed (ten coimplants per group), and the results represent mean ± SD (error bars) from 20 coimplants per group. Statistical significance was determined by the Student's t test. *$p < 0.01$.

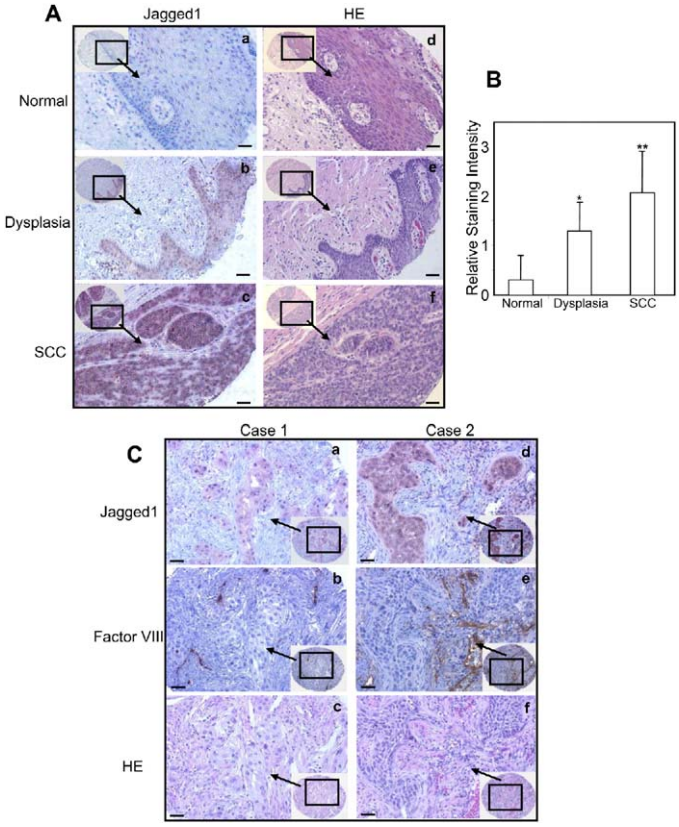

Figure 7. Jagged1 expression is highly elevated and correlated with human microvessel formation in human HNSCC tissues

A and B: Jagged1 expression is highly elevated in HNSCC. Human TMA sections were stained with anti-Jagged1 (1:50). The level of Jagged1 was scored based on staining intensity of normal or dysplastic epithelial cells and tumor cells: 0 = negative; 1 = weak; 2 = moderate; 3 = strong. A total of 350 samples (259 SCCs, 17 normal epithelial tissues, 18 dysplasias, and 56 adjacent muscle and connective tissues) were scored. Of note, some tissue samples were lost during staining. The average scores were calculated from each group, and statistical significance was determined by the Student's t test. Error bars represent standard deviation. *$p < 0.01$, dysplasias versus normal epithelial tissues; **$p < 0.01$, SCC versus dysplasias or normal epithelial tissues. Scale bar, 25 μm.

C: Jagged1 expression was correlated with human microvessel formation in HNSCC. TMA sections were stained with anti-factor VIII (1:200) and anti-Jagged1 (1:50), respectively. Scale bar, 25 μm.

sentative examples of staining for Jagged1 in normal epithelial tissues, dysplasias, and SCC are shown in Figure 7A. Mean Jagged1 staining intensity was significantly increased in human HNSCC compared with dysplasias and normal epithelial tissues ($p < 0.01$). Also, we observed that Jagged1 staining was modestly stronger in dysplasias than that in normal epithelial tissues (Figures 7A and 7B). However, we did not find that there was significant difference in Jagged1 staining between clinical or pathological stages of HNSCC. To determine whether

Table 1. The correlation between the expression of Jagged1 and factor VIII in HNSCC[a]

	Factor VIII				
Jagged1	+	++	+++	++++	Total
0	0	1	0	0	1
+	7	37	3	0	47
++	0	38	50	6	94
+++	0	8	43	31	82

Tissue microarray (TMA) sections were stained with anti-factor VIII and anti-Jagged1, respectively. The microvessel content was scored based on staining intensity and area of human factor VIII: + = weak; ++ = moderate; +++ = strong; ++++ = very strong. The level of Jagged1 was scored as described in Figures 7A and 7B. A total of 224 SCC samples that could be scored for both Jagged1 and factor VIII expression were utilized for our studies. [a]Pearson correlation coefficient analysis. $p < 0.01$; $r = 0.65$.

there was corelationship between Jagged1 expression and tumor blood vessel formation in HNSCC tumor tissues, TMA was also stained with anti-factor VIII antibodies. Our representative photograph demonstrates that tumor blood vessel formation was significantly increased in tumors with higher expression of Jagged1 compared with tumors with low expression of Jagged1 (Figure 7C). Interestingly, in some samples, we observed that some invasive SCC cells were surrounded by factor VIII-positive cells. Moreover, the statistical analysis also found that Jagged1 expression was correlated with blood vessel intensity in HNSCC tumor tissues (Pearson correlation coefficient; $p < 0.01$) (Table 1). Our results suggest that HNSCC cells may utilize the Notch signaling pathway to promote tumor angiogenesis in vivo and that the level of Jagged1 expression may be associated with the development of HNSCC.

Discussion

Our studies demonstrate that contact-dependent Notch signaling triggered by the MAPK signaling pathway plays a critical role in tumor angiogenesis. Although some early works reported that Notch signaling might inhibit angiogenesis, recently, elegant genetic and molecular studies suggested that Notch signaling plays a critical role in angiogenesis during development (Krebs et al., 2000; Lawson et al., 2002; Duarte et al., 2004; Krebs et al., 2004; Gale et al., 2004). In Notch or Jagged1 mutant mouse embryos, vasculogenic formation of the head, yolk sac, and intersomitic vessel was unaffected. Instead, there was a failure to reorganize these rudimentary vessels into large vessels and branching capillaries (Xue et al., 1999; Krebs et al., 2000). These results suggest that Notch signaling may not be required for vasculogenesis but is essential for physiological angiogenesis. Currently, how Notch signaling in angiogenesis is activated during development is not clear. Our results suggest that the upregulation of Jagged1 expression in SCC cells may provide a unique mechanism to control neovascularization. The Jagged1-expressing SCC cells may not only promote, but also guide angiogenesis, thus providing nutrients to support the development and growth of HNSCC.

Importantly, we identified a novel mechanism of tumor angiogenesis mediated by the crosstalk between tumor cells and ECs via the Notch and MAPK signaling pathways. Several secreted proangiogenic factors, including IL-8 and VEGF, have been found to be induced by the MAPK or Akt signaling pathways (Jung et al., 2002; Sparmann and Bar-Sagi, 2004). Interestingly, the knockdown of Jagged1 expression significantly abolished proangiogenic effects of the SCC cell coculture upon HGF stimulation. Moreover, the depletion of the high basal level of Jagged1 in unstimulated SCC cells also abolished the proangiogenic effects of the SCC cell coculture. Our results suggest that contact-dependent Notch signals play a critical role in endothelial differentiation. However, our findings did not rule out the possible contribution of the proangiogenic effects of IL-8 or other secreted proangiogenic factors. In fact, we observed that conditioned media from HGF-treated SCC cells modestly induced endothelial differentiation in vitro. After the conditioned medium was concentrated, we found that it could more strongly promote endothelial differentiation (data not shown), suggesting that secreted proangiogenic factors function in a dose-dependent manner. Importantly, we found that Jagged1 expression was also induced by other growth factors, including TGFα and EGF, via the activation of MAPK, suggesting that the crosstalk between tumor cells and ECs could be triggered by these growth factors.

Genetic studies have demonstrated that there is a crosstalk between the Notch and MAPK signaling pathways in cell fate determination using a model system of *C. elegans* and *Drosophila* (Tsuda et al., 2002; Kumar and Moses, 2001; Shaye and Greenwald, 2002; Yoo et al., 2004). For example, Berset et al. (2001) and Yoo et al. (2004) have demonstrated that the Notch signaling pathway inhibited the MAPK signaling pathway through the induction of multiple negative regulators of MAPK during *C. elegans* vulval development (Shaye and Greenwald, 2002). On the contrary, it was also found that the EGFR-MAPK activation could downregulate Notch via endocytosis during *C. elegans* vulval development. These studies suggest that the coordination of signals from different pathways must be precisely integrated for cell fate specification during development. It has been found that Notch can function either as a tumor suppressor or as an oncogene, depending on the cellular context (Maillard and Pear, 2003). According to the results from *C. elegans*, the inhibition of MAPK by Notch provides a reasonable explanation for Notch-mediated tumor suppression in some cases.

However, it is possible that the principal findings elucidated by studying *C. elegans* vulval development may not be applicable to human cancer development. Studies by Weijzen et al. (2002) have found that Notch was also activated in Ras-transformed cells in which MAPK was constitutively activated. The inhibition of Notch signaling suppressed Ras-mediated transformation. Mailhos et al. (2001) have reported that Dll4 expression was induced during tumor angiogenesis in a mouse model

of xenografted breast adenocarcinoma by unknown mechanisms. Additionally, Notch activation has been found to be associated with the development of several human cancers such as salivary gland carcinoma, leukemia, and invasive pancreatic cancer (Maillard and Pear, 2003). Our studies presented here defined a unique mode of interplay between Notch and MAPK signaling pathways, providing a molecular explanation for Notch-mediated oncogenic transformation. While we found that constitutive activation of Notch signaling alone in tumor cells was not sufficient to induce angiogenesis, the upregulation of Jagged1 was capable of initiating crosstalk between tumor cells and ECs, thereby stimulating tumor angiogenesis and guiding tumor invasive growth. In support of our findings, our immunostaining studies have found that Jagged1 expression was associated with human tumor blood vessel formation. In our immunostaining studies, we did frequently observe that Jagged1-expressing SCC cells were surrounded by microvessels. Compared with normal epithelial tissues, Jagged1 was modestly increased in epithelial dysplasia and more highly expressed in SCC, suggesting that Jagged1 may be associated with the development of SCC. In human cancer development, tumor-associated inflammation has been found to play a critical role in tumor growth and angiogenesis (Sparmann and Bar-Sagi, 2004). In addition to growth factors, it is also possible that Jagged1 or other Notch ligands can be activated by nonspecific inflammatory processes (Guo et al., 2004). In future studies, it will be interesting to explore whether tumor-associated inflammation promotes tumor angiogenesis via the Notch signaling pathway or whether tumor-associated inflammatory cells express the Notch ligands. Nevertheless, our studies suggest that, in addition to the secretion of proangiogenic growth factors, tumor cells may express Notch ligands to guide angiogenesis, which supports tumor growth and progression.

Experimental procedures

Cell culture, retroviral infection, and reagents

SCC cell lines were derived from patients with HNSCC in Dr. Thomas Carey's laboratory at the University of Michigan and were cultured in DMEM supplemented with 10% FBS from Invitrogen (Grand Island, NY). SCC1 was derived from patients with HNSCC in the floor of mouth. SCC5, SCC9, and SCC14A were derived from a patient with HNSCC in the oropharynx (Takebayashi et al., 2000). The human dermal microvascular ECs (cat. CC-0288) were purchased from Cambrex (Walkersville, MD) and were cultured in EGM-2 medium supplemented with growth factors (cat. CC-4147; Cambrex). The chemical inhibitors U0126 (cat. 9903) and LY294002 (cat. 9901) were purchased from Cell Signaling (Beverly, MA). The specific γ-secretase inhibitor IX (cat. 565770) was purchased from Calbiochem (La Jolla, CA). The method for retroviral infection is provided in the Supplemental Data.

Transfection and luciferase reporter assay

Transient transfections were performed by lipofectamine (cat. 18324; Invitrogen) according to the manufacturer's protocol. ECs were plated in a six-well plate overnight and then cotransfected with the Notch luciferase reporter pGA981-6 (Wu et al., 2001) and pRL-TK Renilla luciferase reporter as an internal control. Twenty-four hours after transfection, the transfected ECs were cocultured with SCC/Jag or SCC/V cells for additional 24 hr. Luciferase activities were measured using a dual luciferase system (Promega) as described previously (Zeng et al., 2002a).

Western blot and Northern blot analyses

SCC cells were plated in 10 cm tissue culture dishes the day before treatment. Cells were treated with HGF (40 ng/ml; R&D Systems) for the indicated times. Cells were harvested, and whole-cell extracts were prepared. Western blot analysis was performed as described in the Supplemental Data. For Northern blot analysis, total RNAs were extracted with the Trizol reagents (Invitrogen) according to the manufacturer's protocol. Five micrograms of total RNAs were resolved on 1.5% agarose formaldehyde gels and transferred to nitrocellulose membranes overnight. The membranes were hybridized with ^{32}P-labeled full-length Jagged1 cDNA probes released from pHyTC-Jagged1 plasmids. The probes were prepared with a random-primed labeling kit (Amersham; Arlington Heights, IL) in the presence of [α-32P]dCTP (ICN Pharmaceuticals, Costa Mesa, CA) as described previously (Zeng et al., 2002b).

The endothelial network formation assay in Matrigel and siRNA transfection

The reduced Matrigels (125 μl; BD Systems) were plated in 8-well chamber slides. The chambers were then incubated at 37°C for 30 min to allow the Matrigel to polymerize (Kumar et al., 2004). For coculture assay, 0.8×10^4 SCC cells and 0.8×10^4 ECs were mixed and added to the top of the Matrigel in each well. The chambers were then incubated at 37°C for 24 hr. After incubation, the slides were fixed with methanol and stained with Diff-Quick solution II (Sigma). The slides were examined, and the sprouts were counted from five random fields under a microscope (×200).

Cells were transfected with Jagged1 siRNA (100 μM) or control luciferase siRNA (100 μM) overnight mixed with Oligofectamine (cat. 1225-2-011) diluted in Opti-Mem (Invitrogen) according to the manufacturer's instruction. Thirty-six hours following transfection, cells were treated with HGF for 0, 1, 4, and 8 hr, and the knockdown of Jagged1 expression was confirmed by Western blot analysis. For coculture experiments, siRNA-transfected SCC cells were untreated or treated with HGF for 4 hr and washed with PBS. Afterwards, ECs (0.8×10^4) and siRNA-transfected SCC cells (0.8×10^4) were plated on the Matrigel. The target sequence for Jagged1 (NM_000214) siRNA was 5′-GAACAUCACAUUUACCUUUUU-3′. The target sequence for luciferase is 5′-GCCATTCTATCCTCTAGAG GATG-3′. Both Jagged1 and luciferase siRNAs (cat. 002099-01-20) were synthesized by Dharmacon.

The SCID mouse model of human tumor angiogenesis assay

PLLA (Sigma) scaffolds were prepared as described previously (Nor et al., 2001). 0.5×10^6 HDMECs and 0.5×10^6 SCC9/Jag cells or SCC9/V cells were mixed with the Matrigel and loaded into scaffolds. Four-week-old female SCID mice were purchased from Taconic and anesthetized with ketamine and xylazine. One scaffold containing the mixture of ECs and SCC9/Jag cells and another with ECs and SCC9/V cells were implanted subcutaneously on the left and right flank region of each mouse, respectively. Three or six weeks after transplantation, mice were sacrificed, and the scaffolds were retrieved, immediately measured with calipers, and weighed in an electronic balance. Afterwards, the scaffolds were fixed with 10% buffered formalin and embedded in paraffin. The specimens were sectioned at 4 μM for histological examination (Nor et al., 2001). For BrdU labeling, mice were injected with BrdU labeling reagents (10 ml/kg; cat. 103; Zymad) 2 hr before the scaffolds were retrieved. The care and transplantation of mice was in accordance with the guidelines of the University of Michigan Committee on Use and Care of Animals (UCUCA).

Immunohistochemistry

Human HNSCC high-density TMA was prepared by the University Michigan Head and Neck SPORE Tissue Core with Institutional Review Board approval. Samples (n = 400) from a total of 102 cases with different clinical and pathological stages were arrayed in slides. At least three tissue cores (0.6 mm diameter) from tumor tissues were sampled from each case, and in some cases three tissue cores from adjacent normal tissues were sampled. The TMAs covered the whole spectrum of HNSCC, including normal epithelial tissues, adjacent normal connective tissues, dysplasias, and primary SCC and metastatic SCC. The detailed methods for immunostaining and scoring are described in the Supplemental Data.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/1/13/DC1/.

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