Adrenergic signaling promotes angiogenesis through endothelial cell–tumor cell crosstalk

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Abstract

Angiogenesis is an important factor in invasive tumor growth, progression, and metastasis. Multiple proangiogenic mechanisms are involved in tumor angiogenesis. In this study, we showed that the neurotransmitter norepinephrine upregulated VEGF (VEGFA) expression in breast cancer cells and that the culture supernatant from norepinephrine-treated breast cancer cells promoted the formation of the capillary-like network of endothelial cells. However, the effects of norepinephrine were further enhanced when the endothelial cells were cocultured with breast cancer cells, indicating a critical role of tumor cell–endothelial cell contacts in norepinephrine-induced tumor angiogenesis. Interestingly, norepinephrine dramatically induced the activation of the Notch pathway, which is a cell-contact-mediated intercellular signaling pathway and tightly linked to tumor cell–stromal cell interaction and angiogenesis, in the endothelial cells that had been cocultured with breast cancer cells. Furthermore, the expression of the Notch ligand Jagged 1 was significantly upregulated by norepinephrine at both mRNA and protein levels in breast cancer cells. Inhibitors of β2-adrenergic receptor (β2-AR), protein kinase A (PKA), and mTOR could reverse norepinephrine-induced Jagged 1 upregulation, indicating that the β2-AR–PKA–mTOR pathway participates in this process. Knockdown of Jagged 1 expression in breast cancer cells not only repressed norepinephrine-induced activation of the Notch pathway in cocultured endothelial cells but also evidently impaired the effects of norepinephrine on capillary-like sprout formation. These data demonstrate that tumor angiogenesis mediated by the Jagged 1/Notch intercellular signaling is governed by the norepinephrine-activated β2-AR–PKA–mTOR pathway.

Key Words
- catecholamine
- β2-AR
- Jagged 1
- Notch
- angiogenesis
- breast cancer

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Introduction

Tumor angiogenesis is an intricate process involving the activation of normal quiescent vasculature, formation of new vascular branches, and complicated interactions among tumor cells, endothelial cells, pericytes, and extracellular matrix components. Under normal physiology, angiogenesis is strictly controlled. By contrast, tumor angiogenesis, once initiated, continues indefinitely. An alteration in the balance between proangiogenic and antiangiogenic stimuli turns on the angiogenic switch, which has been considered as an initial and rate-limiting step in malignant conversion and a hallmark of cancer progression (Hanahan & Weinberg 2000, 2011).

Multiple angiogenic growth factors trigger the angiogenic switch. Among these factors, VEGF, as a potent

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endothelial mitogen that induces a rapid and complete angiogenic response, has been implicated in the neovascularization in a wide variety of tumors (Folkman 1971, 2007). Increased production of VEGF by both tumor cells and stromal cells has been associated with angiogenesis in breast cancer. A number of factors are considered to contribute to the upregulation of VEGF expression in breast cancer. Hypoxic microenvironment and hypoxia-inducible factor (HIF), a heterodimeric transcription factor, play significant roles in the induction of the VEGF expression and tumor-associated angiogenesis (Rademakers *et al*. 2008, Semenza 2012). Chronic release of inflammatory cytokines is often accompanied by intense angiogenesis. Multiple lines of evidence indicate that inflammatory cytokines (such as IL1β) are especially important in the regulation of epithelial proliferation and induction of VEGF expression or release in angiogenesis (Jung *et al*. 2003, Voronov *et al*. 2003). Additionally, changes in the tumor microenvironment also positively influence VEGF production. For example, matrix metalloproteinases (MMPs), which are expressed at high levels in tumor tissues, participate in the degradation of the vascular basement membrane and remodeling of the extracellular matrix, facilitating release of sequestered VEGF during angiogenesis (Folkman 2007).

Results from recent studies have indicated that neuroendocrine dynamics and neurotransmitters can manipulate the biobehaviors of tumor and stromal cells and affect tumor angiogenesis (Thaker *et al*. 2006, Lutgendorf *et al*. 2010, Shi *et al*. 2013*a*). Catecholamines influence tumor angiogenesis by inducing the release of proangiogenic factors from tumor cells and by directly modulating the tumor microenvironment. Norepinephrine and epinephrine are potent stimulators of VEGF and vascularization (Thaker *et al*. 2006, Chakroborty *et al*. 2009, Yang *et al*. 2009, Shi *et al*. 2011). It has been reported that chronic restraint stress, which results in high levels of tissue catecholamines, enhanced tumor angiogenesis in primary ovarian tumors by upregulating the expression of VEGF and MMPs, and that a β-blocker reversed stress-enhanced angiogenesis, indicating potential roles of catecholamines and the β-adrenergic receptor (β-AR)-mediated signaling pathway in tumor angiogenesis (Thaker *et al*. 2006). Catecholamines induced the expression of HIF1α (HIF1A) under aerobic conditions and stimulated HIF1A-mediated VEGF expression in human breast cancer cells (Park *et al*. 2011). It has also been reported that catecholamines induced the secretion of endogenous proinflammatory cytokines (such as IL1β, IL6, and IL8) and production of MMPs in tumor cells (Elenkov & Chrousos 2002, Johnson *et al*. 2005, Lutgendorf *et al*. 2008, Shahzad *et al*. 2010, Shi *et al*. 2010).

The tumor microenvironment, where multiple stromal and tumor cells interact, is a predominant determinant of tumor angiogenesis. Interplay between the tumor and angiogenic endothelial cells significantly influences tumor growth and angiogenesis. An evolutionarily conserved Notch signal that regulates short-range intercellular interactions has been implicated in the control of vasculogenesis and angiogenesis (Li & Harris 2005, Zeng *et al*. 2005, Funahashi *et al*. 2008). In mammals, there are four Notch receptors (Notch 1–Notch 4) and five Notch ligands (Jagged 1 and 2 and Delta-like 1, 3, and 4). Binding of the ligands expressed by neighboring cells initiates a series of proteolytic cleavages of the Notch receptors on adjacent cells by the disintegrin metalloprotease (ADAM) and γ-secretase and subsequent nuclear translocation of the Notch intracellular domain (NICD) (Ranganathan *et al*. 2011). In the nucleus, the NICD associates with a transcription factor, immunoglobulin J kappa recombination signal sequence-binding protein (RBPJ), and regulates the transcription of numerous target genes. There is increasing evidence that the Notch signaling pathway plays key roles in physiological angiogenesis and tumor angiogenesis (Dufraine *et al*. 2008, Benedito *et al*. 2009). Results from several studies have indicated that the Notch ligands expressed in tumor cells can activate endothelial cells expressing the Notch receptors and regulate tumor angiogenesis (Zeng *et al*. 2005, Li *et al*. 2007).

In this study, we show that catecholamines upregulate the expression of Jagged 1 in breast cancer cells through the β2-AR–PKA–mTOR pathway and promote capillary-like sprout formation of vascular endothelial cells in an intercellular interaction-dependent manner. Knockdown of Jagged 1 expression in breast cancer cells not only remarkably inhibited the activation of Notch signaling in vascular endothelial cells but also impaired NE-induced formation of the capillary network. These results indicated that tumor angiogenesis mediated by the Notch intercellular signaling is governed by the catecholamine-activated β2-AR pathway.

Materials and methods

Cell culture and treatment

Human breast cancer cell lines MCF-7, MDA-453, and MDA-231, mouse breast cancer cell line 4T1, and human umbilical vein endothelial cells (HUVECs) were obtained from the American Type Culture Collection (Rockville, MD, USA). The authentication of these cell lines was performed

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by short tandem repeat analysis. MCF-7, MDA-453, and 4T1 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μg/ml). MDA-231 cells were maintained in DMEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). HUVECs were cultured in F12K medium supplemented with 10% FBS, 0.1 mg/ml heparin sulfate, 0.05 mg/ml endothelial cell growth factor supplement (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin. All of the cell lines were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C. Cells were incubated overnight in a serum-free medium and then treated with 1 μM or 10 μM norepinephrine (Sigma), 10 μM epinephrine (Sigma), 10 μM isoproterenol (Sigma), or 1 μM salmeterol (Tocris, Ellisville, MO, USA). For treatment with the inhibitors, cells were pretreated with 5 μM WP1066, 500 nM rapamycin, 10 μM H89, 10 μM propranolol, 10 μM ICI 118 551, or 10 μM ATEN.

Western blot

The following antibodies were used for immunoblotting: antibodies against Jagged 1 (Cell Signaling, Danvers, MA, USA), p-STAT3 (Cell Signaling), STAT3 (Cell Signaling), p-mTOR (Cell Signaling), mTOR (Cell Signaling), p-p70S6 kinase (Thr389, Cell Signaling), and glyceraldehyde-3-phosphate dehydrogenase (Sungene Biotech, Tianjin, China). All experiments were performed in duplicate.

Angiogenesis *in vitro*

HUVECs were infected with the recombinant lentiviruses expressing green fluorescent protein (LV-GFP, GeneChem, Shanghai, China) and subsequently plated in a 96-well plate (5×10^3 cells/well) coated with a thin layer of Matrigel (BD Biosciences, San Jose, CA, USA). Then, the HUVECs were cultured with the supernatant of breast cancer cells treated with vehicle or norepinephrine. Alternatively, 5×10^3 breast cancer cells and 5×10^3 HUVECs were mixed and added to the top of the Matrigel in the presence or absence of norepinephrine. The sprouts, representing the degree of angiogenesis *in vitro*, were counted in five random low-power fields.

Construction

JAG1 cDNA was amplified from the total RNA of MDA-231 cells by RT-PCR using the specific primers (sense: 5′-CCGGAATTCAGCACCAGCGCGAACAGCAG-3′ and antisense: 5′-CCGCTCGAGTACGATGTACTCCATTCG-GTTTAAGCTCTG-3′) and cloned into pcDNA3 expression vector (Invitrogen) designated pcDNA3/*JAG1*.

The promoter region (1230 bp) of the human *JAG1* gene was PCR amplified from the genomic DNA of MDA-231 cells (sense primer: 5′-CCGCTCGAGGCAGGACATA-CCTACTATTAGGGCC-3′ and antisense primer: 5′-CCC-AAGCTTAAGGACCCGGAGAGCCCGTCT-3′) with Power Pfu DNA polymerase and ligated immediately upstream of a firefly luciferase gene in the pGL3 basic vector (Promega) designated pGL3/*JAG1*.

Transient transfection

The siRNAs specifically targeting *JAG1* (5′-CGCCAAAUC-CUGUAAGAAU-3′ or 5′-GUGACAAAGAUCUCAAUUA-3′) or *ADRB2* (5′-CAUCGUGUCCUUCUACGUU-3′) were chemically synthesized by RIBOBIO (Guangzhou, China). Scramble siRNA (RIBOBIO) was used as a control. MDA-453, MCF-7, and MDA-231 cells were transfected with the siRNAs targeting *JAG1* using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. MDA-453 cells were transfected with the *ADRB2* siRNA.

Conventional and quantitative RT-PCR

MDA-453, MDA-231, and MCF-7 cells were treated with 1 or 10 μM norepinephrine. The total RNA was isolated from cells using TRIzol reagent (Invitrogen) at the indicated time points. cDNAs were synthesized using a reverse transcription kit (Promega) following the manufacturer's instructions. Conventional RT-PCR was employed to detect the expression of the *VEGF* mRNA induced by norepinephrine (with primers listed in Supplementary Table S1, see section on supplementary data given at the end of this article). Amplification of β-actin (with the primers listed in Supplementary Table S1) was used as the control. The expressions of Jagged 1 and β-actin in MDA-231, MDA-453, 4T1, and MCF-7 cells were detected by real-time RT-PCR (with the primers listed in Supplementary Table S1) as described in our previous study (Shi *et al*. 2013*b*). The experiments were performed three times independently.

Luciferase reporter assays

Notch luciferase reporter plasmid pGA981-6 is a generous gift from Professor Hua Han (the Fourth Military Medical

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University of China). The HUVECs were cotransfected with pGA981-6 and pRL-TK reporter plasmids using Lipofectamine 2000 (Invitrogen). After transfection for 48 h, the transfected cells were cocultured with breast cancer cells in the presence or absence of norepinephrine. MDA-231 cells were cotransfected with pGL3/*JAG1* and pRL-TK reporter plasmids. After transfection for 48 h, the transfected cells were incubated overnight in a serum-free medium and then treated with 1 μM norepinephrine (Sigma). The luciferase activities were measured using a dual luciferase assay kit (Promega) according to the manufacturer's instructions. The experiments were performed three times independently.

Immunohistochemistry

Paraffin-embedded tissue sections were dewaxed and gradually hydrated. Endogenous peroxidase activities were quenched using 3% hydrogen peroxide. After antigen retrieval, the sections were incubated with the rabbit polyclonal antibody against CD31 (Abcam, Cambridge, MA, USA) followed by washing with PBS. Then, the sections were incubated with a HRP-conjugated goat anti-rabbit antibody. The color was developed by incubation with 3,3′-diaminobenzidine solution. Photomicrographs were taken using an Olympus microscope BX53. Omission of the primary antibody and substitution by nonspecific rabbit IgG at the same concentration were used as negative controls.

In vivo tumor model

Six-week-old female BALB/c mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). Animals were housed according to standard animal protocols. The experiment was approved by the Animal Care and Use Committee of the Institute of Basic Medical Sciences. A total of 0.1 ml 4T1 cell suspension (10^6 cells/ml) was injected subcutaneously into the right upper flanks of mice. Mice were divided into two groups randomly and each group contained five mice. They received PBS or isoproterenol (10 mg/kg; Sigma) by daily i.p. injections. After 18 days of tumor implantation, mice were killed and tumors dissected. The expression of Jagged 1 in the tumor tissues at the mRNA and protein levels was detected by quantitative RT-PCR and western blotting respectively. The expression of CD31 in tumor tissues was assayed by immunohistochemistry.

Statistical analysis

All data are expressed as mean ± s.d. Student's *t*-test was used for comparisons between two groups. For comparisons of three or more groups, one-way ANOVA followed by the Bonferroni *post hoc* test was used. $P<0.05$ was considered statistically significant.

Results

Tumor cell–endothelial cell contacts reinforce norepinephrine-induced angiogenesis

To test the effects of norepinephrine on *in vitro* angiogenesis, we treated HUVECs with 10 μM norepinephrine over 24 h. The results indicated that norepinephrine alone had no evident effect on the formation of capillary-like tubular structures (Fig. 1A). Several recent studies have demonstrated that norepinephrine upregulates the expression of VEGF and promotes tumor angiogenesis in a variety of tumors (Thaker *et al.* 2006, Shi *et al.* 2011). Increased production of VEGF has been linked to angiogenesis in breast cancer. Norepinephrine-induced effects are mainly mediated through β2-AR in tumor cells. We examined the expression of β2-AR at both the mRNA and protein levels in human breast cancer cell lines. The expression of β2-AR was relatively high in MDA-453 cells and low in MCF-7 cells. In MDA-231 cells, β2-AR was at an intermediate level (Fig. 1B). When MDA-453 cells were treated with norepinephrine, the expression of *VEGF* mRNA was markedly upregulated (Supplementary Fig. S1A, see section on supplementary data given at the end of this article). As VEGF is considered to be one of the most important regulators of angiogenesis, we treated HUVECs with the supernatant from norepinephrine–stimulated MDA-453 cells and observed the formation of the capillary-like network of HUVECs. To facilitate the observation, HUVECs were infected with the lentiviruses expressing GFP (LV-GFP). The data in Fig. 1C (upper panel) and D indicate that the supernatant from norepinephrine-treated MDA-453 cells induced the formation of capillary-like tubular structures by HUVECs after incubation for 18 h. An even more dramatic proangiogenic effect was observed after coculture of HUVECs with MDA-453 cells in the presence of norepinephrine, as demonstrated by measuring the number of endothelial cell sprouts and evaluating the integrity of capillary-like tubular structures (Fig. 1C, lower panel and D). The results indicate that tumor cell–endothelial cell contacts

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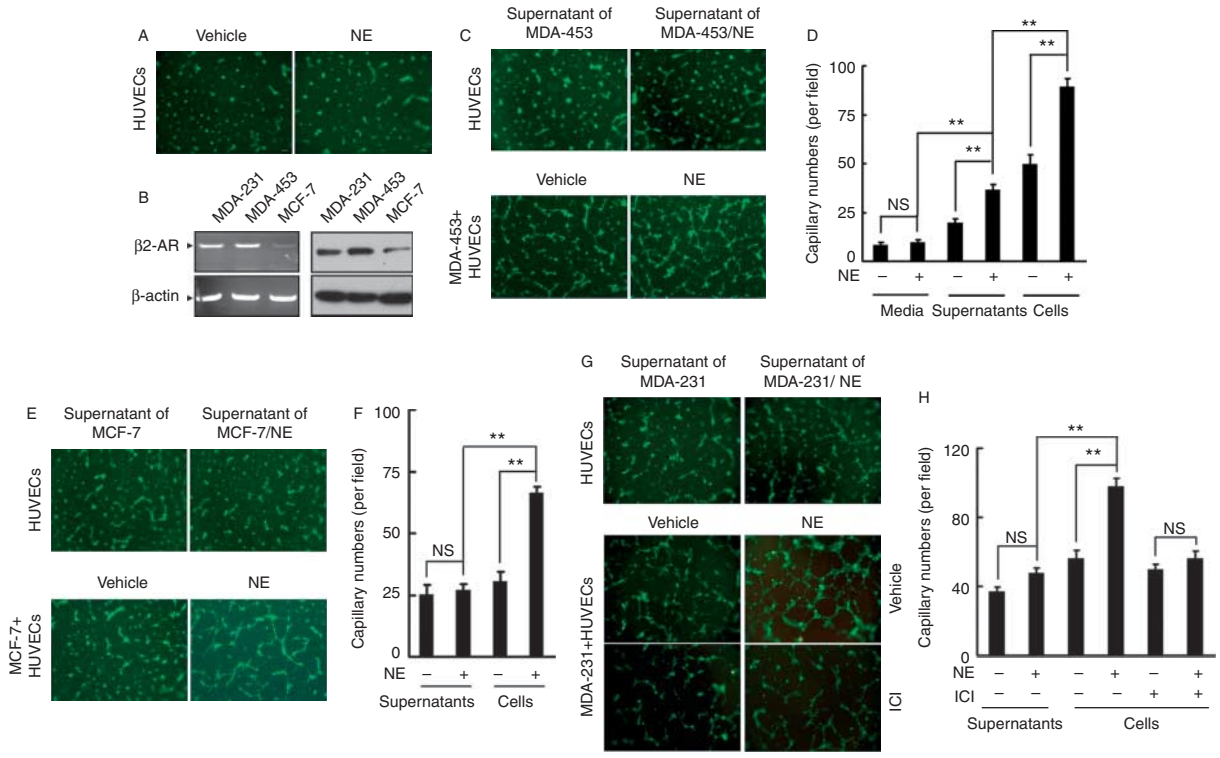

Figure 1

Tumor cell–endothelial cell contacts reinforce norepinephrine-induced angiogenesis. (A) HUVECs infected with the lentiviruses expressing GFP were plated in a 96-well plate coated with Matrigel and treated with 10 μM norepinephrine (NE) for 24 h. The formation of capillary-like tubular structures was observed. (B) The expression of β2-AR in MDA-231, MDA-453, and MCF-7 cells was analyzed by RT-PCR and western blot. (C) HUVECs were incubated with the supernatant of norepinephrine-treated MDA-453 cells (upper panel) or cocultured with MDA-453 cells in the presence of norepinephrine (lower panel). (D) The number of capillary-like structures was measured. (E) HUVECs were incubated with the supernatant of norepinephrine-treated MCF-7 cells (upper panel) or cocultured with MCF-7 cells in the presence of norepinephrine (lower panel). (F) The number of capillary-like structures was measured. (G) HUVECs were incubated with the supernatant from norepinephrine-treated MDA-231 cells (upper panel). MDA-231 cells that were pretreated with 10 μM ICI 118 551 (ICI) and HUVECs were mixed and added to the top of the Matrigel in the presence of norepinephrine (middle and lower panels). (H) The capillary-like structures was observed and counted. $^{**}P<0.01$. Results are representative of three experiments.

may be more important in the development of neovasculature in tumors.

To further test the hypothesis, we repeated the experiment using MCF-7 cells. As shown in Fig. 1E and F, the supernatants of norepinephrine-treated MCF-7 cells did not significantly affect the formation of capillary-like tubular structures, although norepinephrine treatment appeared to affect the expression of the *VEGF* mRNA (Supplementary Fig. S1B). However, coculture of HUVECs with MCF-7 cells significantly promoted norepinephrine-triggered formation of the capillary-like network formation (Fig. 1E and F). Similar data were obtained with MDA-231 cells (Fig. 1G, H, and Supplementary Fig. S1C), confirming that interactions between tumor cells and endothelial cells reinforce norepinephrine-induced tumor angiogenesis. The β2-AR-specific inhibitor ICI 118 551 effectively inhibited norepinephrine-induced *in vitro* angiogenesis by HUVECs cocultured with MDA-231 or MDA-453 cells (Fig. 1G, H, and Supplementary Fig. S1D and E). In addition, knockdown of *ADRB2* by specific siRNA in MDA-453 cells also markedly repressed the formation of the capillary-like network induced by norepinephrine (Supplementary Fig. S1D and E), indicating a potential role of norepinephrine-induced β2-AR activation in cell–cell contact-dependent angiogenesis.

Activation of the Notch pathway in vascular endothelial cells is triggered by norepinephrine-induced Jagged 1 expression in breast cancer cells

Accumulating evidence indicates that tumor cell–stromal cell interaction may be influenced by the Jagged/Notch pathway (Zeng *et al.* 2005, Sethi *et al.* 2011, Lu *et al.* 2013, Xing *et al.* 2013, Zender *et al.* 2013). Results from recent studies have indicated that the Notch signaling pathway is directly involved in physiological and pathological

angiogenesis (Dufraine *et al*. 2008, Benedito *et al*. 2009). To investigate whether communications between tumor and endothelial cells induce the activation of Notch signaling, HUVECs were transfected with pGA981-6 that harbors the hexamerized RBP-J binding site and cocultured with MCF-7 or MDA-453 cells in the presence of norepinephrine. Then luciferase activities were analyzed. As shown in Fig. 2A and B, norepinephrine induction did not evidently affect the Notch reporter activities in HUVECs cultured alone. However, when HUVECs were cocultured with MCF-7 or MDA-453 cells, the luciferase activities were dramatically elevated in response to norepinephrine. Norepinephrine induced the Notch reporter activities very quickly in HUVECs cocultured with MCF-7 cells, reaching a maximum at 3 h (Fig. 2A). The induction of the Notch reporter activities was relatively slow in HUVECs cocultured with MDA-453 cells, but norepinephrine treatment elicited a much greater response at 12 h (Fig. 2B). These results indicate that interactions between vascular endothelial cells and breast cancer cells in response to norepinephrine trigger the activation of the Notch signaling pathway.

Activation of the Notch signaling is mediated by interactions of bordering cells via cell–cell contact through the membrane-associated Notch receptors and ligands (Ranganathan *et al*. 2011). It is known that vascular endothelial cells express Notch receptors (Villa *et al*. 2001, Shawber & Kitajewski 2004). Noticeably, the upregulation of the Notch ligand Jagged 1 is correlated with poor prognosis for breast cancer patients (Reedijk *et al*. 2005, 2008, Dickson *et al*. 2007, Sethi *et al*. 2011). To test whether the Notch pathway activation by norepinephrine in the cocultured endothelial cells is associated with the upregulation of Jagged 1 expression in breast cancer cells, we examined the expression of Jagged 1 after treatment of breast cancer cells with catecholamines. Cells treated with vehicle were used as controls. Treatment of MDA-231 cells with norepinephrine, epinephrine, or isoproterenol resulted in a significant increase in the expression of Jagged 1 in a time-dependent manner (Fig. 3A, B, and C). Similar results were also observed in MDA-453 cells and mouse breast cancer cell line 4T1. In MCF-7 cells, upregulation of Jagged 1 expression occurred quickly (within 1 h), but declined subsequently (Fig. 3D, E, and F). No responses were observed in the control cells (results not shown).

To examine whether enhanced expression of Jagged 1 induced by catecholamines in breast cancer cells is associated with the activation of the Notch pathway in vascular endothelial cells, the expression of Jagged 1 was knocked down by the specific siRNA targeting Jagged 1 in MDA-231 cells (Fig. 3G). Figure 3H shows that norepinephrine stimulation remarkably promoted the Notch reporter activities in HUVECs that were cocultured with MDA-231 cells transfected with control siRNA. However, knockdown of Jagged 1 expression in MDA-231 cells greatly repressed norepinephrine-induced Notch reporter activities in HUVECs. These results indicate that norepinephrine modulates the Jagged 1/Notch signaling in a tumor cell–endothelial cell contact-dependent manner.

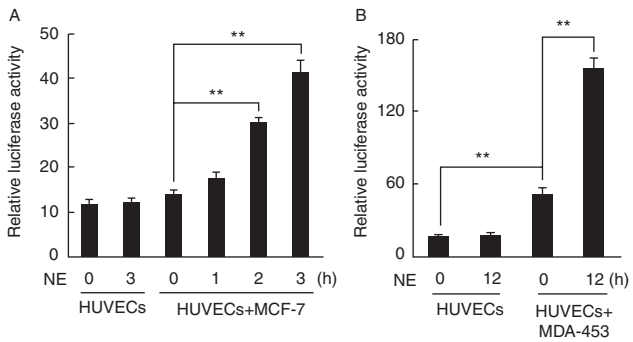

Figure 2
Tumor cell–endothelial cell contacts enhance norepinephrine-induced Notch reporter activities in HUVECs. HUVECs were cotransfected with pGA981-6 and pRL-TK reporter plasmids. The transfected cells were cocultured with MCF-7 (A) or MDA-453 cells (B) in the presence of 10 μM norepinephrine. The luciferase activities were measured. $^{**}P<0.01$. Results are representative of three experiments.

Expression of Jagged 1 in breast cancer cells is upregulated by catecholamines through activation of the β2-AR–PKA–mTOR pathway

To determine whether the upregulation of Jagged 1 expression occurs at the transcription level, we examined *JAG1* mRNA expression after norepinephrine stimulation by real-time RT-PCR. As shown in Fig. 4A and B, the level of the *JAG1* mRNA was increased by greater than fourfold within 1 h after norepinephrine stimulation, reaching a maximum (approximately eightfold) at 3 h, in MDA-231 cells. Upregulation of *JAG1* by norepinephrine showed a definite time-dependent property. The effect of norepinephrine was also analyzed in MDA-453, 4T1, and MCF-7 cells (Fig. 4C, D, and Supplementary Fig. S2A, see section on supplementary data given at the end of this article).

To explore the molecular mechanisms by which norepinephrine stimulates Jagged 1 expression, MDA-231 cells were cotransfected with pGL3/*JAG1* and pRL-TK

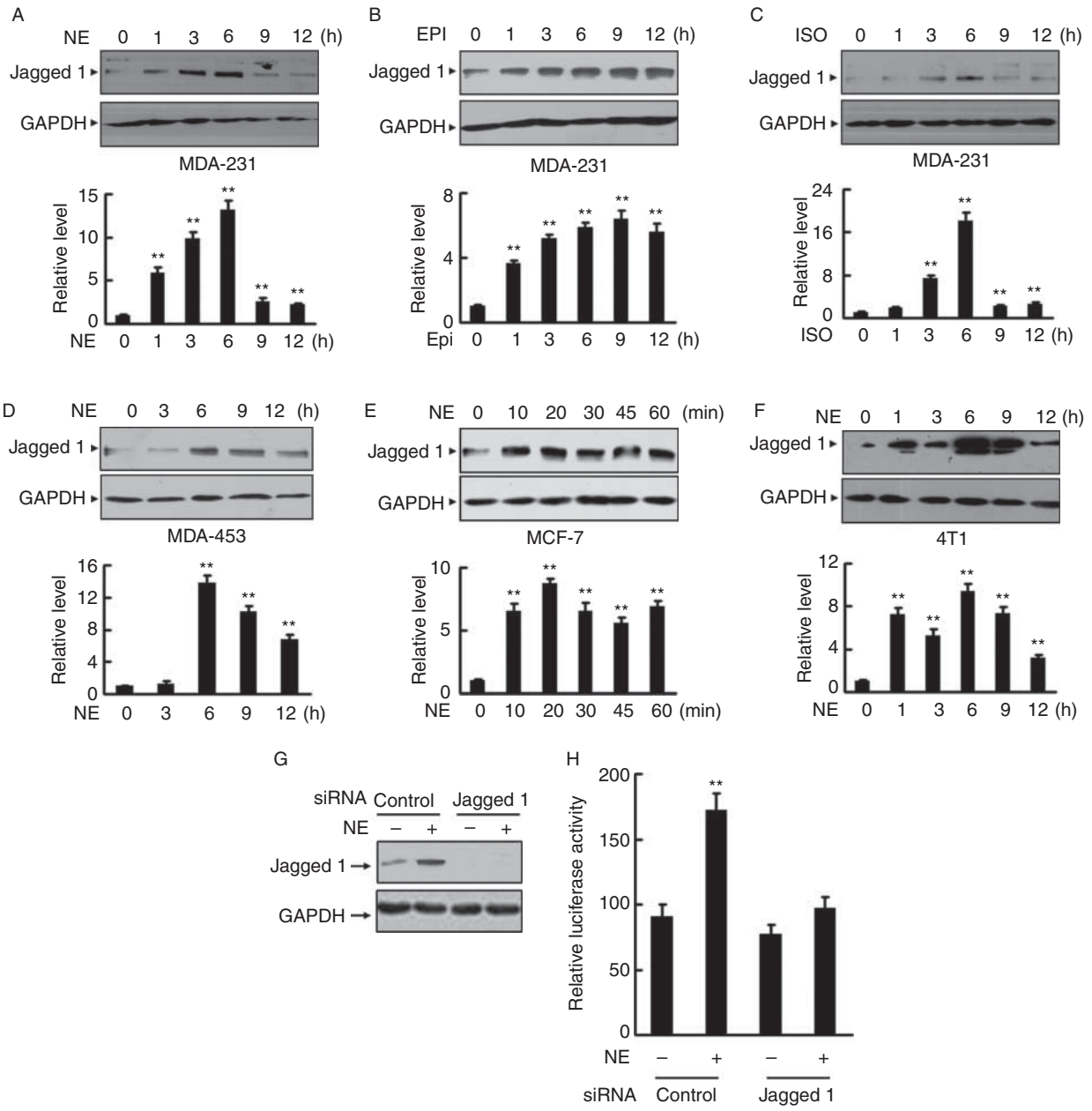

Figure 3

Catecholamines stimulate upregulation of Jagged 1 in breast cancer cells. (A, B, C, D, E, and F) MDA-231, MDA-453, MCF-7, and 4T1 cells were treated with 10 μM norepinephrine or 10 μM epinephrine (EPI) or 10 μM isoproterenol (ISO). The expression of Jagged 1 was analyzed by western blotting and densitometry assays at the indicated time points. (G) MDA-231 cells were transfected with the siRNA against Jagged 1 or control siRNA and the expression of Jagged 1 was analyzed. (H) HUVECs were cotransfected with pGA981-6 and pRL-TK reporter plasmids. Then, cells were cocultured with the transfected MDA-231 cells in the presence of 10 μM norepinephrine. The Notch reporter activities were analyzed by luciferase assays. $^{**}P<0.01$. Results are representative of three experiments.

reporter plasmids and the effects of norepinephrine on *JAG1* promoter activity was assessed by luciferase assays. The results indicate that the luciferase activities began to rise at 0.5 h after norepinephrine treatment. At 2 h after exposure, the *JAG1* promoter activities reached a peak and then decreased after 6 h (Supplementary Fig. S2B), indicating that norepinephrine stimulation could directly induce the transactivation of the *JAG1* promoter. The β-AR inhibitor propranolol and the β2-AR-specific inhibitor ICI 118 551 strongly inhibited norepinephrine-induced

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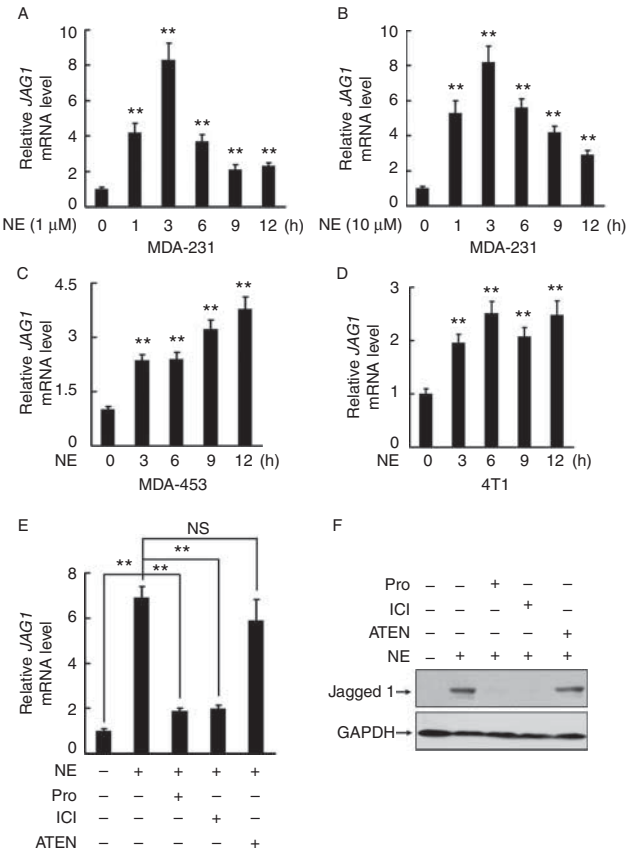

Figure 4
Norepinephrine induces upregulation of Jagged 1 at the transcriptional level through the β2-AR pathway. (A, B, C, and D) MDA-231, MDA-453, and 4T1 cells were treated with 1 or 10 μM norepinephrine. The expression of *JAG1* mRNA was detected by real-time RT-PCR at the indicated time points. (E and F) MDA-231 cells were pretreated with 10 μM propranolol (Pro) or 10 μM ICI 118 551 (ICI) or 10 μM ATEN. Then, cells were stimulated with 10 μM norepinephrine. The expression of *JAG1* mRNA and protein were analyzed by real-time RT-PCR (E) and western blotting (F). $^{**}P<0.01$. Results are representative of three experiments.

Jagged 1 transcription and protein expression, but the β1-AR antagonist ATEN had no effect (Fig. 4E and F). The effect of the β2-AR pathway on norepinephrine-induced Jagged 1 upregulation was also analyzed in MDA-453, MCF-7, and 4T1 cells (Supplementary Fig. S2C).

We observed that 4T1 cells expressed β2-AR and that the selective β2-AR agonist salmeterol upregulated the expression of Jagged 1 at both the mRNA and protein levels (Supplementary Fig. S2D and E). To examine whether β-AR agonist induces the expression of Jagged 1 *in vivo*, we treated mice bearing 4T1 tumors with isoproterenol (10 mg/kg) daily for 18 consecutive days. Expectedly, the Jagged 1 expression in the 4T1 tumor tissues was prominently upregulated at both transcriptional and protein levels (Supplementary Fig. S2F and G). Additionally, tumor microvessel density was also greatly enhanced (Supplementary Fig. S2H and I).

Results from recent studies have indicated that aberrant activation of the STAT3 and mTOR pathways is highly involved in the regulation of Jagged 1 expression (Sansone *et al.* 2007, Studebaker *et al.* 2008, Ma *et al.* 2010). Our previous studies have demonstrated that catecholamines stimulate STAT3 activation in breast (Shi *et al.* 2011) and gastric cancer cells (Shi *et al.* 2010, 2013*b*). Results shown in Fig. 5A indicate that norepinephrine induced persistent phosphorylation of STAT3 in MDA-231 cells. In addition,

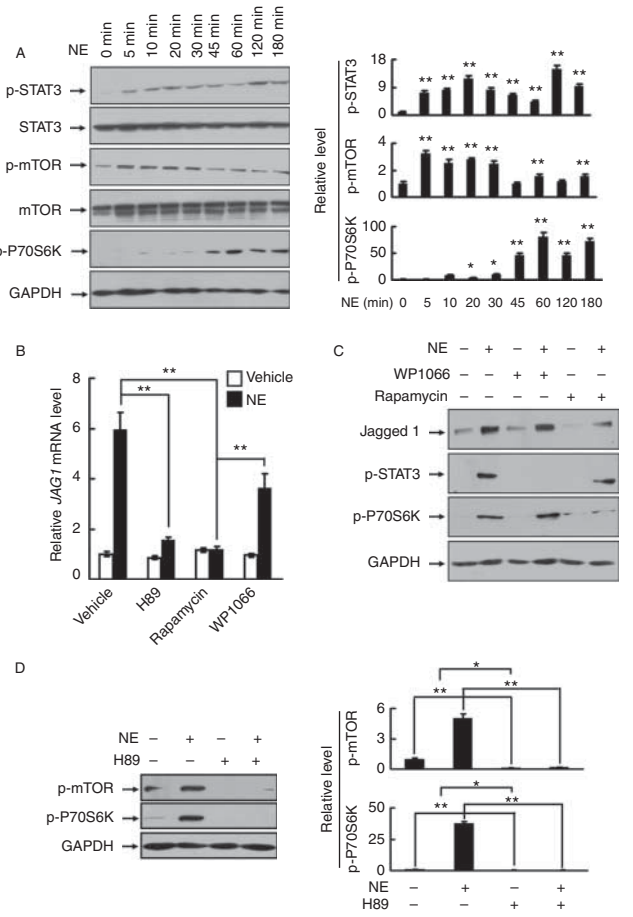

Figure 5
Expression of Jagged 1 in breast cancer cells is upregulated by catecholamines through activation of the β2-AR–PKA–mTOR pathway. (A) MDA-231 cells were treated with 10 μM norepinephrine. Phosphorylation of STAT3, mTOR, and P70S6K was analyzed by western blotting and densitometry assays at the indicated time points. (B) MDA-231 cells were pretreated with rapamycin (500 nM) or WP1066 (5 μM) or H89 (10 μM). The expression of the *JAG1* mRNA induced by norepinephrine was analyzed by real-time RT-PCR. (C) MDA-231 cells were pretreated with WP1066 and rapamycin. The expression of the Jagged 1 protein and phosphorylation of STAT3 and P70S6K induced by norepinephrine were analyzed. (D) MDA-231 cells were pretreated with H89 and then stimulated with norepinephrine. Phosphorylation of mTOR and P70S6K was analyzed. $^{*}P<0.05$; $^{**}P<0.01$.

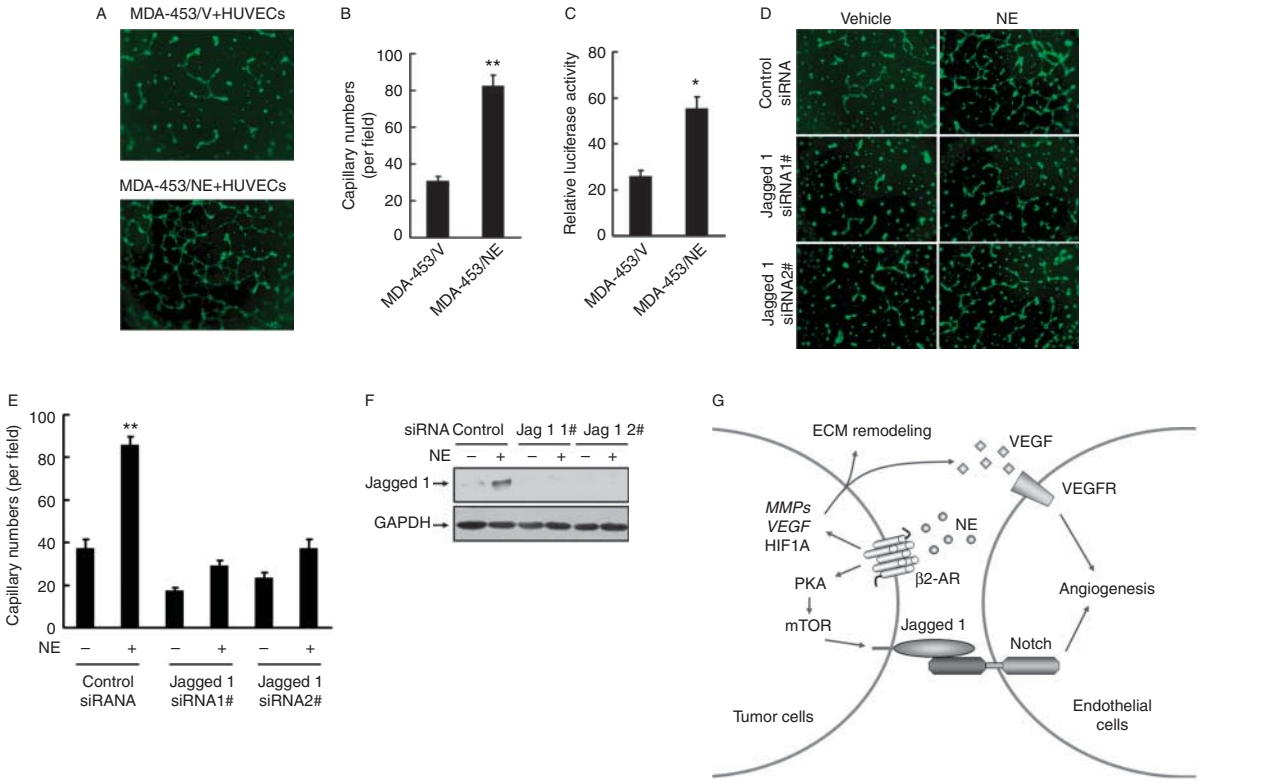

Figure 6

Upregulation of Jagged 1 is associated with norepinephrine-induced breast tumor angiogenesis. MDA-453 cells were pretreated with norepinephrine (MDA-453/NE) or a vehicle (MDA-453/V) followed by washing with PBS. Cells were gently detached with enzyme-free cell dissociation buffer and then placed on HUVECs. (A) The formation of capillary-like structures was observed. (B) The number of capillary-like structures was measured. (C) HUVECs were cotransfected with pGA981-6 and pRL-TK reporter plasmids and then cocultured with norepinephrine-treated MDA-453 cells. The Notch reporter activities were analyzed by luciferase assays. (D) MDA-453 cells were transfected with *JAG1* siRNA and then cocultured with HUVECs. The formation of capillary-like structures in the presence of norepinephrine was observed. (E) The number of capillary-like structures was measured. (F) The expression of Jagged 1 in the transfected cells was analyzed. $*P<0.05$; $**P<0.01$. Results are representative of three experiments. (G) Catecholamines induce tumor angiogenesis by orchestrating multiple molecular mechanisms. Catecholamines upregulate the expression of *VEGF*, *MMP*s, and HIF1A, resulting in extracellular matrix (ECM) degradation, tissue remodeling, and tumor angiogenesis. Catecholamines also induce tumor angiogenesis by upregulating Jagged 1 expression in breast cancer cells through the β2-AR–PKA–mTOR pathway and subsequently activating the Notch signaling pathway in adjacent endothelial cells.

mTOR and P70S6K, a downstream target of mTOR, were also phosphorylated after norepinephrine stimulation (Fig. 5A). Similar data were obtained with MCF-7 cells (Supplementary Figure S2J). To investigate whether the upregulation of Jagged 1 by catecholamines is through activation of STAT3 and mTOR, cells were treated with the mTOR inhibitor rapamycin and the STAT3 inhibitor WP1066. As shown in Fig. 5B, WP1066 suppressed norepinephrine-induced transcription of the *JAG1* mRNA, whereas the inhibitory effect of rapamycin was much stronger. Western blot analyses indicate that the expression of Jagged 1 protein induced by norepinephrine was also significantly repressed by rapamyin, but the inhibitory effect of WP1066 was only marginal (Fig. 5C). These results indicate that the activation of mTOR may play a predominant role in norepinephrine-induced Jagged 1 expression. As catecholamine stimulation of β2-AR increases the activity of protein kinase A (PKA), we employed an inhibitor of PKA H89 to test whether the activation of mTOR in response to norepinephrine is PKA-dependent. Pretreatment of MDA-231 cells with H89 fully abrogated norepinephrine-induced phosphorylation of mTOR and P70S6K (Fig. 5D). Moreover, norepinephrine-induced transcription of *JAG1* mRNA was also remarkably impaired by H89 (Fig. 5B). It is noticeable that p-mTOR and p-P70S6K are undetectable in the presence of H89. PKA is an important upstream molecule of the mTOR signaling and a downstream molecule of the β2-AR pathway as well. Alteration of mTOR and P70S6K phosphorylation in the presence of H89 could be expectable. However, suppression

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of mTOR and P70S6K activation by H89 was significantly more potent in the presence of norepinephrine than in the absence of norepinephrine (Fig. 5D), indicating that the β2-AR/PKA system may be involved in the activation of the mTOR pathway induced by norepinephrine. These results indicate that the expression of Jagged 1 in breast cancer cells is upregulated by catecholamines through the activation of the β2-AR–PKA–mTOR pathway.

Upregulation of Jagged 1 associated with norepinephrine-induced breast tumor angiogenesis

To further explore whether catecholamine-induced Jagged 1 upregulation affects tumor angiogenesis, MDA-453 cells were pretreated with norepinephrine (MDA-453/NE) or a vehicle (MDA-453/V) followed by washing with PBS. Cells were gently detached with enzyme-free cell dissociation buffer, harvested, and then placed on HUVECs. Fig. 6A and B show that coculture remarkably induced the formation of the capillary-like network. Compared with MDA-453/V cells, coculture of HUVECs with MDA-453/NE cells more effectively promoted angiogenesis *in vitro*. The adhesions of the tumor cells (appearance in bright-field microscopy and disappearance in fluorescence-field microscopy) to the endothelial cells were clearly visualized by fluorescence microscopy (Supplementary Fig. S3A, see section on supplementary data given at the end of this article). Although both MDA-453/NE and MDA-453/V cells could adhere to the endothelial cells, MDA-453/NE cells more strongly stimulated the formation of mature capillary networks (Fig. 6A and B). In addition, the Notch-dependent transcription activities were also greatly enhanced in the HUVECs cocultured with MDA-453/NE cells (Fig. 6C). Similar results were obtained by coculturing HUVECs with norepinephrine-treated MDA-231 cells (Supplementary Figures S3B, C and D). Enforced expression of Jagged 1 in MDA-231 cells promoted the formation of extensive capillary networks by the endothelial cells in coculture system (Supplementary Figure S4A, see section on supplementary data given at the end of this article). Knockdown of Jagged 1 expression markedly abolished norepinephrine-induced Jagged 1 upregulation in MDA-453 cells, impaired the effects of norepinephrine on formation of capillary-like structures by the HUVECs in coculture systems (Fig. 6D, E, and F), and inhibited norepinephrine-induced Notch pathway activation (Supplementary Fig. S4B). The results were confirmed using MCF-7 cells transfected with Jagged 1 siRNA (Supplementary Figures S4C, D, and E). Together, these data support the hypothesis that upregulation of Jagged 1 by norepinephrine in breast cancer cells promotes tumor angiogenesis through Notch intercellular signaling.

Discussion

A growing body of evidence indicates that Notch ligands are important in development and carcinogenesis. It has been reported that Notch ligands are upregulated in several human malignant diseases (Ranganathan *et al.* 2011). Upregulation of Jagged 1 has been correlated with reduced disease-free survival and increased incidence of relapse in human breast cancer (Reedijk *et al.* 2005, 2008, Sethi *et al.* 2011). Enhanced Jagged 1 expression in breast cancer cells induces epithelial-to-mesenchymal transition, which is a key step toward cancer metastasis, by repressing the E-cadherin expression (Leong *et al.* 2007). Results from a recent study have shown that the upregulation of Jagged 1 promoted osteolytic bone metastasis by activating the Notch pathway in bone cells (Sethi *et al.* 2011). Additionally, aberrant activation of Notch signaling has also been confirmed by demonstration of the accumulation of NICD in a wide variety of breast cancer cell lines and breast cancer tissues. Increased RBP-J-dependent Notch signaling has been shown to be sufficient to transform normal breast epithelial cells and attenuation of Notch signaling could reverse the transformed phenotypes of breast cancer cells. The results of these studies indicate the important roles of the Notch ligands and Notch signaling pathway in breast cancer (Stylianou *et al.* 2006).

Results from previous studies including ours have indicated that β2-AR is overexpressed in breast cancer tissues and certain types of breast cancer cell lines (Powe *et al.* 2011, Shi *et al.* 2011). Catecholamine-induced activation of β2-AR modulates the expression of numerous prosurvival, invasion, and metastasis genes through multiple signaling cascades (Cole & Sood 2012, Shi *et al.* 2013*a*). In this study, we show that catecholamines stimulate the upregulation of Jagged 1 in breast cancer cells, leading to the activation of the Notch pathway in vascular endothelial cells and tumor angiogenesis. The inhibitors of β2-AR, PKA, and mTOR can reverse norepinephrine-induced Jagged 1 upregulation, indicating that the β2-AR–PKA–mTOR pathway plays a central role in mediating norepinephrine-induced Jagged 1 upregulation and Notch signaling activation. We noticed that in the absence of norepinephrine the Notch reporter activities were significantly higher in HUVECs cocultured with MDA-453 cells than in HUVECs alone (Fig. 2B), indicating that the Notch signaling pathway in HUVECs may be activated by adjacent MDA-453 cells expressing high levels

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of β2-AR and Jagged 1. An interesting finding is that norepinephrine-induced formation of vascular-like structures strongly depends on tumor cell–endothelial cell contacts, in addition to the proangiogenic effect of VEGF, which is also induced by norepinephrine stimulation, indicating that a juxtacrine stimulatory mechanism may also contribute to norepinephrine-induced tumor angiogenesis and that neighboring tumor cells may serve as a source for activators of the Notch receptors on endothelial cells. Numerous types of cells exist in the tumor microenvironment, including lymphocytes, neutrophils, macrophages, fibroblasts, and myofibroblasts besides tumor and endothelial cells. It is not known yet whether catecholamines influence the Jagged 1/Notch signaling triggered by contact between tumor cells and other types of nontumor cells. The roles of catecholamines in modulating tumor microenvironment and reprogramming of tumor cell phenotypes deserve further exploration.

It is known that the VEGF pathway is absolutely required for the early stages of developmental angiogenesis and that Notch signaling is also essential for sprouting angiogenesis. These two pathways are perhaps the most important mechanisms in the regulation of tumor angiogenesis. Results from recent studies have indicated that the VEGF pathway interacts at several levels with Notch signaling (Thurston & Kitajewski 2008, Thomas *et al*. 2013), whereas complex crosstalk between Notch and VEGFRs modulates Notch signaling and its effects on angiogenic activity. Our results indicate that Jagged 1 upregulation in breast cancer cells and Notch activation in endothelial cells occurred very quickly in response to norepinephrine. The proangiogenic effect triggered by tumor cell–endothelial cell contacts was much stronger than that of norepinephrine-induced tumor cell supernatants, indicating that an intercellular signaling mechanism predominates in norepinephrine-induced tumor angiogenesis in addition to the VEGF pathway.

VEGF has been confirmed to be an important therapeutic target in cancer (Folkman 2007). Results from various preclinical studies have indicated that blockade of the VEGF pathway effectively inhibits tumor growth and angiogenesis. VEGF inhibitors have been recognized to be potentially useful agents in several major cancers. However, not all tumors are responsive to the VEGF inhibitors and some tumors initially responded but later became unresponsive, indicating the existence of other angiogenic signaling pathways or compensatory mechanisms (Bergers & Hanahan 2008, Loges *et al*. 2009, Sennino & McDonald 2012). A more detailed understanding of the complex nature of the angiogenic process and its regulation will provide an abundant source of molecular targets for anti-angiogenic therapy (Sennino & McDonald 2012). The elucidation of the mechanisms that govern the pathological communications between the tumor and endothelial cells will help to improve anti-angiogenic strategies.

Results from several recent studies have indicated that stress-induced catecholamines upregulate the synthesis of many proangiogenic factors, such as VEGF, through the β2-AR-mediated signaling pathway in a variety of malignant tumor cells and induce angiogenesis in the tumor tissues (Thaker *et al*. 2006, Madden *et al*. 2011, Park *et al*. 2011). It has also been shown that norepinephrine stimulates the production of MMP2 and MMP9, which mediate extracellular matrix degradation and tissue remodeling and induce angiogenesis, through the β2-AR signaling pathway in tumor cells and tumor-associated macrophages (Cole & Sood 2012). During chronic stress, which is a common concern across the course of the cancer trajectory and considered to be the '6th vital sign' (Howell & Olsen 2011), substantial amounts of norepinephrine and epinephrine (EPI) are produced owing to the activation of the hypothalamic–pituitary–adrenal axis and sympathetic nervous system (Lutgendorf *et al*. 2010). In this study, we have demonstrated that catecholamines trigger the angiogenic switch by upregulating Jagged 1 expression in breast cancer cells through the β2-AR–PKA–mTOR pathway, resulting in the activation of the Notch signaling pathway in adjacent endothelial cells (Fig. 6G). These results together with previous findings indicate that activation of the β2-AR signaling pathway by catecholamines may be a key event in the tumor angiogenesis cascade.

The results of recent experimental studies have demonstrated the promising antitumor activities of blockers for β-ARs or antagonists for neurotransmitters. Several retrospective clinical studies also provide evidence that β-blocker usage is associated with improved relapse-free survival in patients with breast cancer (Powe *et al*. 2010, Barron *et al*. 2011, Melhem-Bertrandt *et al*. 2011). Our results reveal that the β2-AR-mediated signaling pathway plays critical roles in manipulating tumor angiogenesis by integrating multiple mechanisms. Elucidating the interplay of neuroendocrine and angiogenesis signaling pathways may open new windows for developing novel drugs or therapeutic strategies against cancers.

Supplementary data

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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