Blocking Notch in Endothelial Cells Prevents Arteriovenous Fistula Failure Despite CKD

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ABSTRACT
Neointima formation causes the failure of 60% of arteriovenous fistulas (AVFs) within 2 years. Neointima-forming mechanisms are controversial but possibly linked to excess proinflammatory responses and dysregulated Notch signaling. To identify how AVFs fail, we anastomosed the carotid artery to the internal jugular vein in normal and uremic mice and compared these findings with those in failed AVFs from patients with ESRD. Endothelial cells (ECs) of AVFs in uremic mice or patients expressed mesenchymal markers (FSP-1 and/or α-SMA) and exhibited increased expression and nuclear localization of Notch intracellular domain compared with ECs of AVFs in pair-fed control mice. Furthermore, expression of VE-Cadherin decreased, whereas expression of Notch1 and -4, Notch ligands, the downstream transcription factor of Notch, RBP-Jκ, and Notch target genes increased in ECs of AVFs in uremic mice. In cultured ECs, ectopic expression of Notch ligand or treatment with TGF-β1 triggered the expression of mesenchymal markers and induced endothelial cell barrier dysfunction, both of which were blocked by Notch inhibition or RBP-Jκ knockout. Furthermore, Notch-induced defects in barrier function, invasion of inflammatory cells, and neointima formation were suppressed in mice with heterozygous knockdown of endothelial-specific RBP-Jκ. These results suggest that increased TGF-β1, a complication of uremia, activates Notch in endothelial cells of AVFs, leading to accelerated neointima formation and AVF failure. Suppression of Notch activation could be a strategy for improving AFV function in uremia.


The preferred vascular access for patients with ESRD is an arteriovenous fistula (AVF). Unfortunately, their patency is estimated to be only 60% after 1 year.1–3 AVF failure develops mainly from vascular stenosis created by neointima formation and/or thrombosis.4,5 Hyperplasia of the neointima is created by inflammatory and proliferative responses, but the role of endothelial damage in the process of neointima development is unknown.

One candidate for triggering neointima formation is the dysfunction of endothelium. In the cardiovascular system, the endothelium is not only a barrier between the circulating blood and vascular smooth muscle cell, but, also, it releases mediators that regulate vascular tone, vessel growth, platelet function, and coagulation.6 Moreover, endothelial damage/dysfunction interferes with these functions. Endothelial cells (ECs) could influence the fate of an AVF. For example, in patients with CKD, biomarkers of endothelial dysfunction (vWF and vascular cell adhesion molecule-1) are expressed in ECs, and the capacity for endothelial repair is decreased.7 However, the relationships among CKD-induced changes in ECs and neointima formation in AVFs are poorly defined.

A candidate mediator of endothelial cell dysfunction is Notch signaling. It is initiated by binding to its ligands with triggering proteolytic cleavage of the

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transmembrane receptor by γ-secretase and release of the Notch intracellular domain (NICD). After translocation of NICD into the nucleus, it associates with the DNA binding protein recombinant binding protein-Jk (RBP-Jk) plus coactivators and initiates expression of target genes, such as α-smooth muscle actin (α-SMA) or the Hes family proteins. Not surprisingly, the Notch pathway is involved in multiple aspects of vascular development, including regulation of endothelial function and control of endothelial mesenchymal transition in the heart valve. Increased Notch signaling has also been associated with neointima formation and arteriovenous malformations.

Our goal was to identify the role of Notch signaling during the development of neointima formation in AVFs. We show that neointima formation in AVFs of mice with CKD is associated with the development of an abnormal phenotype of ECs: there are expressions of some mesenchymal markers in ECs. In response to CKD, the expressions of α-SMA and fibroblast-specific protein 1 (FSP-1) in ECs from AVFs are increased, but there is reduced endothelial barrier function. These changes in ECs were found to be activated by the Notch pathway in AVFs placed in mice with CKD. Thus, we identified a pathway that begins with activation of Notch in endothelial cells and leads to impaired barrier function of the endothelium and infiltration of inflammatory cells, ultimately resulting in the formation of a neointima. Stimulation of the Notch/RBP-Jk signaling was increased by CKD. The pathway has the potential for identifying Notch-induced changes in the function of ECs that could be a target for preventing neointima formation in AVFs, the Achilles heel of the hemodialysis patients.

RESULTS

In ESRD Patients, Mesenchymal Markers Are Expressed in the Endothelium of Failed AVFs

In normal arteries, α-SMA–positive cells were presented in the media, whereas positive staining of vWF was found only in the endothelium (Figure 1A). In AVFs from ESRD patients, vWF was detected on the surface of the neointima (Figure 1B). However, α-SMA was also expressed in the endothelial layer of AVFs that were characterized as having severe neointima formation (Figure 1B). Double staining of vWF and α-SMA in AVFs from ESRD patients confirmed that endothelial cells expressed vWF plus the mesenchymal marker α-SMA (Figure 1C, upper panel). FSP-1, another mesenchymal marker, was found in the endothelium of failed AVFs from ESRD patients (Figure 1D). There was no colocalization of α-SMA and vWF in microvessels of the adventitium (Figure 1C, lower panel). These results show that endothelial cells of the neointima of AVFs from ESRD patients express the mesenchymal cell marker α-SMA.

CKD Enhances Endothelial Expression of Mesenchymal Markers and Neointima Formation in Mouse AVFs

The levels of BUN, creatinine, and TGF-β1 were all elevated in this mouse model of CKD (Figure 2, A–C). In AVFs created in mice with CKD, there was substantially more neointima formation versus results in AVFs of nonuremic, pair-fed mice (Figure 2D). Other than the increase in neointima in AVFs placed in CKD mice, α-SMA was expressed in the endothelial layer (Figure 2F). Double staining showed colocalization of the endothelial marker (CD31) and α-SMA (Figure 2F). These observations are similar to the findings in AVFs from ESRD patients; notably, the endothelium of AVFs from control mice did not stain positively for α-SMA (Figure 2F). Our results
show that advanced CKD leads to the expression of mesenchymal markers in ECs of AVFs from uremic mice.

In AVFs from CKD mice, vascular endothelial (VE)-Cadherin mRNA was reduced compared with results in the AVFs from control mice (Figure 2G). \( \alpha \)-SMA mRNA was significantly increased in AVFs from CKD versus control mice (Figure 2H), and >95% of the VE-Cadherin–positive cells were located in the endothelium. These cells also stained the endothelial marker CD31 positively (Supplemental Figure 1).

We conclude that virtually all VE-Cadherin–positive cells are endothelial cells, and that VE-Cadherin is downregulated in mice with CKD.

**CKD Activates Notch Signaling in AVFs from ESRD Patients and Uremic Mice**

Because Notch signaling regulates cell fate determination and differentiation and is activated in CKD and other kidney diseases, we found that the expression of intracellular domain of Notch1 (N1ICD) in ECs in AVFs from ESRD patients (Figure 3A). By double staining of N1ICD with \( \alpha \)-SMA or vWF, we found that N1ICD was expressed in both neointima and ECs of failed AVFs of ESRD patients (Figure 3, B and C). In fact, N1ICD colocalized with the endothelial marker vWF (Figure 3C). Similar findings were present in AVFs from CKD mice (data not shown).

Other than an increased expression of N1ICD in the nuclei of ECs, there was a dramatic upregulation in the mRNAs of the Notch target genes, Hey2 and Hes1, compared with results in AVFs from sham-control mice (Figure 3D). We also found that Notch1 and -4 and their ligands (Jagged1 and DLL4) were increased in AVFs from CKD mice versus results from control mice (Supplemental Figure 2). Likewise, immunostaining revealed enhanced N1ICD and RBP-Jk expression in the endothelium of AVFs from CKD mice (Figure 3, E and F).

**Notch Activation Induces the Expression of Mesenchymal Markers in ECs**

To examine whether ligand-induced Notch activation affects mesenchymal marker expression in ECs, we cocultured ECs that had been infected with adenovirus to express Notch ligand (AdJagged1) or a control vector in a 1:1 ratio. The response to increased expression of the Notch ligand, Jagged1, included nuclear translocation of N1ICD in both ECs expressing Jagged1 ECs and ECs in contact with Jagged1-expressing cells (Figure 4A). In addition, overexpression of Jagged1 induced \( \alpha \)-SMA and FSP-1 expressions in both groups of cells (Figure 4, B and C).

To determine the relationship between Notch activation and expression of \( \alpha \)-SMA and FSP-1, we expressed Jagged1 in ECs or treated them with TGF-\( \beta \)1 in the presence or absence of a \( \gamma \)-secretase inhibitor, DAPT (N-[N-(3,5-difluorophenacetyl)-l-Ala]-S-phenylglycine t-butyler ester). In TGF-\( \beta \)1–treated cells,
with control). Similarly, there was increased migration of bone marrow cells through the EC monolayers in response to Jagged1 overexpression or TGF-β1 treatment. RBP-Jk KO significantly blocked the increase in transendothelial leakage (Figure 5C).

In AVFs from Mice with CKD, RBP-Jk Knockdown in the Endothelium Suppresses Infiltration of Inflammatory Cells

To examine how endothelial barrier function changes in AVFs, we created RBP-Jk<sup>flox/+</sup>/VE-Cadherin-Cre<sup>+</sup> transgenic mice that do not express the RBP-Jk gene in ECs. Unfortunately, these mice only survive for ~14 days, and, therefore, we isolated ECs from these mice to study how the absence of RBP-Jk affects EC function. In ECs isolated from RBP-Jk KO mice, mRNA levels of Hes1 and -5 were decreased versus results in ECs from WT mice (Figure 6A). Because the Hes1 protein in the endothelium of the transgenic mice, RBP-Jk<sup>flox/+</sup>/VE-Cadherin-Cre<sup>+</sup>, was decreased, we conclude that knockdown of RBP-Jk suppresses the expression of its target genes (Figure 6B). In addition, there was infiltration of macrophages (Mac2<sup>+</sup>) and mononuclear cells (CD45<sup>+</sup>) into AVFs in WT mice with CKD. This infiltration was inhibited in AVFs placed in RBP-Jk knockdown mice (Figure 6, C and D).

RBP-Jk Knockdown Suppresses CKD-Induced Neointima Formation in AVFs

We evaluated the effect of RBP-Jk knockdown on mesenchymal marker expression in the endothelium of AVFs created in CKD mice. Both WT and RBP-Jk<sup>flox/+</sup>/VE-Cadherin-Cre<sup>+</sup> mice with CKD were studied. The expression of α-SMA in the endothelium of AVFs from RBP-Jk knockdown mice was significantly decreased versus results in WT mice with CKD (Figure 7A). The barrier function of the endothelium in AVFs was impaired. There was leakage of Evans Blue dye in AVFs created in RBP-Jk<sup>flox/+</sup>/VE-Cadherin-Cre<sup>+</sup> mice versus results in control mice (Figure 7B).

We compared neointima formation in AVFs from RBP-Jk<sup>flox/+</sup>/VE-Cadherin-Cre<sup>+</sup> and WT control mice (both with CKD). RBP-Jk knockdown in ECs suppressed the CKD-induced neointima formation: there was an increase in the lumen and the lumen/intima ratio in RBP-Jk<sup>flox/+</sup>/VE-Cadherin-Cre<sup>+</sup> mice versus results in WT CKD mice (Figure 7, C–E). These results indicate that inhibition of Notch signaling can suppress both CKD-induced endothelial barrier dysfunction and the formation of a neointima.

DISCUSSION

Endothelial dysfunction, a hallmark of CKD, can begin in the early stages of CKD. In mice with advanced CKD, we found that Notch signaling in ECs is activated by cell–cell interaction,

Figure 3. The expressions of Notch signaling mediators are increased in AVFs from ESRD patients and CKD mice. (A) Notch activation (N1ICD) in human AVFs was detected in ECs (red arrows) and neointima cells (yellow arrow) but not smooth muscle cells of the media (blue arrow). (B) Immunofluorescence staining of N1ICD and α-SMA in human AVFs. DAPI, 4',6-diamidino-2-phenylindole. (C) Serial sections from human AVFs were stained with N1ICD and vWF (green). DAPI staining is shown in blue. (D) Density analysis of the immunostaining of N1ICD and α-SMA or FSP-1 expressions (**P<0.05, compared with control).
leading to the expression of mesenchymal proteins in ECs of the AVFs. One possible trigger of Notch activation is TGF-β1 in uremic mice. In support of this possibility, we found that treating ECs with TGF-β1 stimulated expression of N1ICD. It was followed by endothelial dysfunction and interruption of the EC barrier function, resulting in infiltration of inflammatory cells into the AVFs. Notably, this sequence of events in our mouse model of CKD is similar to results in failed AVFs from patients with ESRD. Both are associated with endothelial dysfunction and neointima formation (Figures 1 and 2). Specific novel results that we uncovered include evidence that Notch signaling is a critical component of the pathogenesis of endothelial barrier dysfunction. It promotes expression of mesenchymal markers in EC, supporting neointima formation, as well as Notch.8,27,28 In cultured epithelial cells, it has been reported that TGF-β1a and Wnt, as well as Notch.8,27,28 In cultured epithelial cells, it has been reported that TGF-β1 stimulates the expression of the Notch ligand Jagged1 and induces epithelial–mesenchymal transition.29 Reportedly, activated Notch1 or -4 can cause similar responses in cultured ECs.30 It also has been reported that expression of soluble Jagged1 interferes with Notch signaling and can reduce the neointima formation induced by balloon injury.31 The present results indicate that CKD plays a role in Notch-induced responses, because it increases the expression of the Notch receptors in AVFs and the expression of the Notch target gene, Hes1, in AVFs (Figure 3B). Our results provide the first evidence for a mechanism by which CKD activates Notch signaling in AVFs, especially in the endothelium (Figure 3).

RBP-Jκ is the major transcription factor that responds to activation of Notch signaling that leads to expression of mesenchymal markers in ECs, such as α-SMA, calponin, and N-cadherin.32–34 This finding was confirmed in cultured ECs with Jagged1 overexpression or exposure to TGF-β1, also stimulated the expression of mesenchymal markers in ECs of the AVFs.

The endothelial monolayer of cells plays a crucial role in vascular homeostasis.15 After vascular injury or EC loss, vascular permeability increases, which promotes the influx of neutrophils and monocytes into the vessel.16,17 These inflammatory cells are involved in neointima cell migration and proliferation.18 Alternatively, a CKD-induced increase in reactive oxidative stress leads to dysfunction of the endothelium and impaired barrier function.19,20 How CKD induced events affecting the AVF is complicated.21,22 Our results indicate that neointima formation in AVFs of ESRD patients or mice with CKD increased expression of mesenchymal cell markers (α-SMA and FSP-1) and decreased VE-Cadherin in ECs (Figures 1 and 2). These changes cause leakage of the endothelium (Figure 5), promoting infiltration of bone marrow–derived inflammatory cells into AVFs (Figure 6C). The inflammatory cells secrete factors that stimulate smooth muscle cell proliferation, which has been reported for MCP-1,23,24 SDF-1,25 or other chemokines after balloon- or stent-induced endothelial dysfunction.26 Likewise, in a vein graft model, we found that bone marrow–derived FSP-1–positive cells can increase smooth muscle cell proliferation and neointima formation.18

We recognize that certain mesenchymal markers (e.g., α-SMA and collagen I) are regulated in non-ECs by multiple intracellular signaling pathways, including TGF-β1 and Wnt, as well as Notch.8,27,28 In cultured epithelial cells, it has been reported that TGF-β1 stimulates the expression of the Notch ligand Jagged1 and induces epithelial–mesenchymal transition.29 Reportedly, activated Notch1 or -4 can cause similar responses in cultured ECs.30 It also has been reported that expression of soluble Jagged1 interferes with Notch signaling and can reduce the neointima formation induced by balloon injury.31 The present results indicate that CKD plays a role in Notch-induced responses, because it increases the expression of the Notch receptors in AVFs and the expression of the Notch target gene, Hes1, in AVFs (Figure 3B). Our results provide the first evidence for a mechanism by which CKD activates Notch signaling in AVFs, especially in the endothelium (Figure 3).

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Figure 4. Activation of Notch pathway in ECs induces expression of mesenchymal markers. (A–C) Lung ECs were infected with AdJagged1 or control vector (AdVector) and cocultured for 24 hours before immunostaining; Jagged1-expressing cells (red) were also positive for (A) N1ICD, (B) α-SMA, or (C) FSP-1 (nuclei were labeled with DAPI). (D) Western blot analysis shows that Notch activation was associated with increased expression of α-SMA and FSP-1. ECs were infected with AdJagged1 or treated with TGF-β1 for 48 hours with or without pretreatment of DAPT (10 μM). The loading control was β-actin. Representative data from three repeated experiments are shown. C, control; DAPI, 4′,6-diamidino-2-phenylindole; Jag, Jagged.
because there was expression of mesenchymal proteins and an increase in EC permeability with more transendothelial migration of bone marrow cells. The key role of Notch signaling also showed that RBP-Jk knockdown in ECs suppresses expression of mesenchymal proteins, as well as leakage of the endothelium and the infiltration of inflammatory cells (Figures 4 and 5). These changes limited neointima formation in AVFs (Figures 6 and 7).

In mammalian cells, there are four Notch receptors (Notch1, -2, -3, and -4) and five Notch ligands (DLL1, -3, and -4 and Jagged1 and -2). All are expressed in ECs except DLL3,35,36; Notch1 and -4 receptors are expressed by ECs.8 This finding is relevant, because Notch1 is the primary functional Notch receptor during developmental angiogenesis;12 also, dysfunction of Notch4 is associated with arteriovenous malformations.37,38 Interestingly, in the AVFs from mice with CKD, the most robust increase in mRNAs was from Jagged1 and Notch1 versus other Notch receptors and their ligands (Supplemental Figure 2). Therefore, we used them and the transcription factor RBP-Jk to study Notch signaling. Because RBP-Jk conducts all Notch signaling, we cannot exclude the involvement of activated Notch4 or even other Notch ligands as contributors to EC dysfunction. Studies of transgenic mice with KO of specific Notch receptors or their ligands will be needed to clarify whether other Notch receptors or ligands affect CKD-induced AVF failure (Figure 8).

We conclude that Notch activation is a critical step in the progressive failure of AVFs in mice. The processes that we uncovered are accelerated by CKD, and, specifically, TGF-β1, a circulating cytokine associated with CKD, can stimulate Notch activation and expression of mesenchymal markers in ECs in AVFs.39,40 These changes contribute to dysfunction of the endothelial barrier, permitting invasion by inflammatory cells and creation of a neointima. Notably, knockout of RBP-Jk blocks this pathway; because Notch signaling is a key activator of a regulatory pathway of endothelial function and neointima formation, we suggest that inhibiting Notch signaling will yield strategies to patency of AVFs in CKD.

CONCISE METHODS

Mice and CKD

All studies were approved by the Institutional Animal Care and Use Committee of Baylor and performed in accordance with National Institutes of Health guidelines. Male mice were kept in a 12-hour light/dark cycle. RBP-Jk floxed mice were provided by K. Susztak (Albert Einstein College of Medicine, NY). VE-Cadherin-Cre transgenic mice were purchased from The Jackson Laboratory. RBP-Jk flox/flox mice were identified by genotyping using the primers 5'-TAAC-TATCTTGAAAGGCTAAAAT-3'; 5'-AAGAGGGCACTTGACTTTTCCAT-3'. PCR products of 850 bp corresponded to the RBP-Jk floxed gene. CKD was induced by subtotal nephrectomy in anesthetized mice as described.39,40 Briefly, mice were fed 20% protein chow, and, after matching for body weight, subtotal nephrectomy was performed in anesthetized mice in a two-step surgery method (ketamine, 125 mg/kg body wt; and xylazine, 6.4 mg/kg body wt). First, the left
kidney was decapsulated to avoid ureter and adrenal damage, and approximately three quarters of the left kidney was removed. During the recovery, the mice were given two doses of buprenorphine (0.1–2.5 mg/kg body wt s.c.) after surgery and 12 hours later. The diet was changed to 6% Protein Rodent Diet Chow (Harlan Teklad, Madison, WI) ad libitum to reduce mortality and limit hypertrophy. Second, the right kidney was removed 1 week later, and after 1 week, the mice with CKD were pair-fed 40% protein chow with sham-operated control mice. The BUN was measured by the Comparative Pathology Laboratory Center at Baylor College of Medicine. The serum creatinine level was detected by using the QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA). After 2–3 weeks, AVFs were created in control and CKD mice; other control and CKD mice underwent sham surgery.

Reagents and Virus
Penicillin, streptomycin, DMEM, and FBS were obtained from Invitrogen Life Technologies (Carlsbad, CA). The γ-secretase Notch inhibitor DAPT was from Calbiochem (San Diego, CA). Human TGF-β1 was purchased from R&D Systems (Minneapolis, MN). The protein assay kit was from Bio-Rad (Hercules, CA), and dextran-FITC was from Sigma-Aldrich (St. Louis, MO). The FSP-1 antibody was obtained from DAKO (Carpinteria, MA), and the rat antibodies against VE-Cadherin and CD31 were from BD Biosciences (San Jose, CA). Antibodies against N1ICD, vWF, and α-SMA antibodies were from Abcam (Cambridge, MA), the RBP-Jκ antibody was obtained from Millipore (Billerica, MA), and Jagged1, Hes1 and -5, TGF-β1, and β-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The fluorescent-700 or -800 secondary antibodies were obtained from Invitrogen (Carlsbad, CA). The full-length Jagged1 recombinant adenovirus was provided by M.J. Post (Maastricht University, The Netherlands).

Human AVF Samples and Mouse AVF Model
We studied samples of AVFs from five hemodialysis patients ranging in age from 58 to 77 years. In each case, samples of failed AVFs were collected at surgery with the approval of the Baylor Institutional Review Board. Mouse AVFs were created as described. Briefly, mice of 12 weeks of age were anesthetized, and the right internal jugular vein was isolated using a dissecting microscope (Leica MZ6; Leica, Germany). Its distal end was clamped and ligated, the common carotid artery was ligated below its bifurcation, and the proximal end was clamped. An end-to-end anastomosis was created using 11–0 nylon suture with an interrupted stitch. After unclamping, patency was confirmed visually. The mice were kept warm after surgery, and the analgesic (buprenorphine) was given two times 12 hours apart. At 2 and 4 weeks after surgery, the mice were anesthetized by intraperitoneal injection and euthanized by perfusing the left ventricle with PBS and 10% formalin for 10 minutes (to maintain the endothelium and morphology of the AVF). AVFs were collected, and slides from 0.5 to 1 mm from the venous anastomosis were collected for hematoxylin/eosin staining.

The neointima and media were defined as the regions between the lumen and the adventitia. The vessel wall thickness was determined by measuring the difference between the area of the lumen and the adventitia. The vessel wall thickness was determined by measuring the difference between the area of the lumen and the adventitia. The vessel wall thickness was determined by measuring the difference between the area of the lumen and the adventitia.

Immunohistochemistry
For histologic analysis, AVFs were perfused through the left ventricle with 10% phosphate-buffered formaldehyde and processed as described. Sections were blocked with 10% goat serum (Vector Laboratories, Burlingame, CA) for 30 minutes and then incubated with.

Figure 6. RBP-Jκ knockdown suppresses inflammatory cell infiltration into AVFs of mice with CKD. (A) mRNA levels of Hes1 and -5 in ECs lacking RBP-Jκ were significantly lower than in WT mice. (B) Immunofluorescent staining of an en face section of the vein of an AVF reveals loss of the Notch target gene, Hes1 (red), in veins from RBP-Jκ knockdown mice compared with results with WT mice. Nuclei were stained with DAPI. (C) RBP-Jκ knockdown in mice with CKD inhibits infiltration of inflammatory cells. After 2 weeks, both Mac2- and CD45-positive cells were detected in AVFs from WT or RBP-Jκ knockdown mice with CKD (n=4 mice per group). The number of positive cells was counted and summarized in D (P<0.05, compared with WT).
primary antibodies (NICD, 1:500; RBP-Jk, 1:500; FSP-1, 1:1000; α-SMA, 1:2000). Sections were washed in 0.5% Tween 20 in PBS and incubated with a biotinylated secondary antibody (Vector Laboratories) at room temperature. After washes in 0.5% Tween 20 in PBS, tissue sections were incubated with an Elite ABC reagent (Vector Laboratories) followed by instructions as described in a peroxidase substrate kit (Vector Laboratories). The sections were counterstained by hematoxylin. For double immunofluorescent staining of samples, fluorescent secondary antibodies were applied to sections; 4',6-diamidino-2-phenylindole was used in counterstaining. Pictures were recorded using a Nikon Eclipse 80i Fluorescence Microscope (Nikon).

**En Face Analysis of AVF**

We analyzed the endothelium in AVFs using an en face technique with immunostaining as described. AVF segments were cut longitudinally, mounted on glass slides with the endothelium facing up, and air dried for 1–2 hours. AVFs were incubated with antibodies against VE-Cadherin and Hes1 followed by immunofluorescent-labeled secondary antibodies (Rockland, Gilbertsville, PA); 4',6-diamidino-2-phenylindole was used to stain nuclei.

**Real-Time RT-PCR**

Total RNAs from control vein or AVF were isolated using the RNeasy Kit (Qiagen, Valencia, CA). Real-time RT-PCR was performed using the Opticon Real-Time RT-PCR Machine (MJ Research, Waltham, MA). The specificity of real-time RT-PCR was confirmed by agarose gel electrophoresis and melting curve analysis. The primers for real-time RT-PCR are listed in Supplemental Table 1.

**Mouse EC Isolation**

Because only small numbers of ECs can be isolated from the AVFs, we studied pathways in cultured ECs from the lung. This study was done because not only are lung ECs plentiful but also, ECs from different tissues maintain major EC characteristics, such as barrier function. We recognize that there may be varied responses to external signals. However, we have found that activated Notch is associated with EC barrier dysfunction in vitro and in vivo. Primary cultures of mouse ECs were isolated from the lungs as described. The lung ECs were maintained in EGM-2 SingleQuot Kit Supplement & Growth Factors (Lonza) plus 20% FBS. ECs with RBP-Jk KO were isolated from RBP-Jk knockdown mice with CKD compared with WT CKD mice. (D and E) In mice with CKD, the neointima area (α-SMA*) and the ratio of neointima to lumen areas in AVFs created in RBP-Jk flox/+ /VE-Cadherin-Cre+ mice are compared with WT mice. The data are presented as mean±SEM (n=5) (*P<0.05, compared with control).

**Figure 7.** RBP-Jk knockdown inhibits CKD-induced neointima formation in AVFs. (A) Double staining of EC markers (CD31) or mesenchymal cell marker (α-SMA) was present in AVFs of WT or RBP-Jk knockdown mice with CKD. Yellow arrows indicate coimmunostaining of CD31 and α-SMA. (B) At 2 weeks after placing the AVF, Evans Blue was administrated intravenously. The vein was perfused with PBS. The intensity of Evans Blue leak was analyzed. The data are presented as mean±SEM (n=4). (C) Hematoxylin & eosin (HE) and α-SMA staining showed that the neointima was significantly smaller (P<0.05), whereas the lumen was larger (P<0.05) in AVFs from RBP-Jk knockdown mice with CKD compared with WT CKD mice. (D and E) In mice with CKD, the neointima area (α-SMA*) and the ratio of neointima to lumen areas in AVFs created in RBP-Jk flox/+ /VE-Cadherin-Cre+ mice are compared with WT mice. The data are presented as mean±SEM (n=5) (*P<0.05, compared with control).
CKD

↑

TGF-β1

↑

Notch activation

RBP-Jκ

Expression of mesenchymal markers

Endothelial barrier dysfunction

Infiltration of inflammatory cells

Neointima formation

AVF failure

Figure 8. Activated Notch signaling in ECs mediates CKD-induced neointima formation. Complications of CKD (such as TGF-β1) induce activation of Notch signaling that stimulates the expression of mesenchymal markers in ECs of AVFs. This leads to EC barrier dysfunction and enhances infiltration of inflammatory cells. These responses promote the formation of neointima and loss of dialysis access. Knockdown of Notch pathway in ECs suppresses CKD-induced neointima formation.

Jκlox/lox/VE-Cadherin-Cre mice within 7 days after birth (mice do not survive after 14 days).

Cell–Cell Interaction

ECs were infected with AdJagged1 or the vector alone for 24 hours. These ECs were trypsinized and cocultured at a 1:1 ratio. After confluence, the ECs were fixed, and immunostaining was performed.

Western Blot Analysis

ECs were lysed in radioimmunoprecipitation assay buffer, and ~20 μg proteins were separated by SDS-PAGE. After transferring to nitrocellulose membranes, antibodies were added.45

Transendothelial Migration Assay

WT ECs or ECs with RBP-Jκ KO were seeded in 24-well Boyden Transwell inserts (2×10⁵/well). After a confluent monolayer was formed, ECs were infected with AdJagged1 or AdFSP-1 for 24 hours. For the transendothelial migration assay, bone marrow cells (2×10⁶) from WT mice were added to the inserts and cultured for 24 hours. The total number of bone marrow cells in the lower chamber was counted. Each experiment was repeated at least three times.

Endothelium Permeability Assay

WT or RBP-Jκ KO ECs treated as described were kept in 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]-DMEM (pH 7.4) for 15 minutes. The HEPES-DMEM was then removed from the top of the well; 100 μl FITC-labeled dextran (12,000 molecular weight; 1 mg/ml in 25 mM HEPES-DMEM) was added to the top of each well. Aliquots (50 μl) were removed from the bottom well at the times specified for each experiment and collected in white 96-well plates. FITC-labeled dextran was measured in a luminescence spectrometer (LS 50B; PerkinElmer) using 480 and 530 nm as the excitation and emission wavelengths, respectively.

In Vivo Analysis of Endothelial Barrier Function of AVFs (Evans Blue Assay)

Before collecting AVFs, 50 μl 5% Evans Blue in saline was injected into the tail vein, and 10 minutes later, mice were perfused with PBS and then 10% neutral buffered formalin through the left ventricle. AVFs were removed and photographed; the contralateral jugular vein was used as control. Evans Blue accumulated in the extracellular matrix after disruption of the endothelium. The density of the stained AVFs was evaluated with ImageJ.

Statistical Analyses

All data are presented as mean±SEM. Results were analyzed using t test when results from two groups were compared or two-way ANOVA when data from over three groups were studied; P<0.05 was considered statistically significant.

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DISCLOSURES

None.

REFERENCES


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