Title: Connexin 43 Across the Vasculature: Gap Junctions and Beyond

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Authors:

Meghan W. Sedovy1,2

Xinyan Leng1

Melissa R. Leaf1,3

Farwah Iqbal1,3

Laura Beth Payne1

John C. Chappell1

Scott R. Johnstone1,4

1 The Fralin Biomedical Research Institute at Virginia Tech Carilion, Center for Vascular and Heart Research, 4 Riverside Circle, Roanoke, Virginia, VA 24016 USA

2 Translational Biology, Medicine, and Health Graduate Program, Virginia Tech, Blacksburg, VA 24061, USA

3 Virginia Tech Carilion School of Medicine, Roanoke, VA 24016 USA

4 Department of Biological Sciences, Virginia Tech, Blacksburg, VA 24060

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Figure: Cx43 expression in the vascular tree

Correspondence: Dr. Scott R. Johnstone. Fralin Biomedical Research Institute at Virginia Tech Carilion, 4 Riverside Circle, Roanoke, VA 24016. Scottrj@vt.edu

Abstract

Connexin 43 (Cx43) is essential to the function of the vasculature. Cx43 proteins form gap junctions that allow for the exchange of ions and molecules between vascular cells to facilitate cell-to-cell signaling and coordinate vasomotor activity. Cx43 also has intracellular signaling functions that influence vascular cell proliferation and migration. Cx43 is expressed in all vascular cell types, although its expression and function vary by vessel size and location. This includes expression in vascular smooth muscle cells (vSMC), endothelial cells (EC), and pericytes. Cx43 is thought to coordinate homocellular signaling within EC and vSMC. Cx43 gap junctions also function as conduits between different cell types (heterocellular signaling), between EC and vSMC at the myoendothelial junction, and between pericyte and EC in capillaries. Alterations in Cx43 expression, localization, and post-translational modification have been identified in vascular disease states, including atherosclerosis, hypertension, and diabetes. In this review, we discuss the current understanding of Cx43 localization and function in healthy and diseased blood vessels across all vascular beds.

Vascular gap junctions

Connexin proteins, first identified in the 1960s, are essential for direct cell-to-cell communication (1-3). Although 21 different connexins have been identified in humans, connexin 43 (Cx43) remains among the most studied isoforms (2). Encoded by the GJA1 gene, Cx43 is highly expressed in many tissues, including the vasculature (4-7). Cx43 has been extensively researched in blood vessels, with expression found in vascular smooth muscle cells (vSMC), endothelial cells (EC), and pericytes, where Cx43 plays a critical role in maintaining normal cellular functions (8). While other connexins, including Cx37, Cx40, and Cx45, all play integral roles in blood vessel homeostasis, this review focuses primarily on the known roles of Cx43.

The primary function of Cx43 is the formation of single membrane channels known as hemichannels, or bi-membrane channels called gap junctions which facilitate the exchange of molecules less than 1000 Da in size between cells (1). Connexin hemichannels, also known as connexons, form when six connexin proteins come together, creating a channel with an aqueous pore inserted into the plasma membrane. Once at the plasma membrane, connexin hemichannels aggregate through directed trafficking at close apposition points between two cells, permitting binding head-to-head to form gap junctions. The gap junctions act as a continuous pore connecting the two cells' cytoplasm (9). Hemichannels can also form plasma membrane channels that connect the cell cytoplasm to the extracellular space (**Figure 1**) (9). Gap junctions facilitate the exchange of molecules such as Ca^{2+}, IP_3, ATP, and cAMP, which have essential intracellular signaling roles in vascular cells (10-13). Hemichannels are also thought to be involved in the release of signaling molecules, including purinergic ATP release (14, 15).

In the vasculature, the role of Cx43 is dependent on the function of the vascular segment in which it is expressed. Cx43 is expressed in large conduit vessel vSMC and regulates cell differentiation and proliferation (7). In smaller contractile arteries, Cx43 gap junctions are essential for cell-to-cell electrical coupling to facilitate coordinated contraction and relaxation of blood vessels (7). Cx43 gap junctions regulate Ca^{2+} signaling between EC in capillaries to coordinate angiogenic responses and mural cell differentiation (16-18). The expression and localization of Cx43 are altered in vascular disease states, including atherosclerotic plaque formation, hypertension, and diabetes which will be discussed in this review (19-21).

The functions of Cx43 are regulated by post-translational modifications, including phosphorylation and S-nitrosylation, which we previously reviewed (22, 23). Phosphorylation primarily occurs in the carboxyl-terminus of Cx43 and can influence trafficking to and from the plasma membrane, gap junction assembly, channel permeability, and protein-protein interactions (22, 24, 25). In the

vasculature, changes in Cx43 phosphorylation have been linked with altered heterocellular communication (e.g., EC to vSMC) at the myoendothelial junction (MEJ) and vSMC proliferation in conduit arteries (26, 27). S-nitrosylation alters the communication between vSMC and EC at the MEJ by regulating the permeability and conductance of Cx43 gap junctions (28). Here, we review the role of Cx43 in the vasculature, including its gap junction-dependent and independent functions, its role across different vascular beds, and its regulation in vascular disease.

Cx43 in conduit vessels

Conduit vessels are large arteries that primarily function to distribute cardiac output from the heart to resistance vessels without significantly altering blood pressure. Conduit arteries are composed of a single layer of EC (intima), multiple vSMC (media) layers, and the surrounding adventitia containing fibroblasts along with immune cells, progenitor cells, nerves, and vasa vasorum capillaries (29). The intima lines the lumen of the vessel and consists of longitudinally oriented EC covering a thick connective tissue subendothelial layer called the internal elastic lamina. Cx43 is expressed in conduit vessels, but this expression is variable based on location, tissue size, and disease state.

Cx43 in conduit vessel EC

Cx40 and, to a lesser extent, Cx37, are expressed in large artery EC. This includes co-expression in rat and mouse aorta and the rat coronary artery (30-34). Cx43 is variably detected in large artery EC, with early studies in rat aortas failing to identify it or demonstrating low expression (33, 35). Later studies found Cx43 expression to be region-specific, with expression in EC limited to branch regions in the thoracic and ascending rat aorta, which experience elevated levels of sheer stress (**Figure 1**) (31, 32, 36). Variable expression of Cx43 is also documented in porcine coronary artery EC and at branch regions of bovine coronary arteries (33). However, several studies did not find Cx43 in rat and human coronary EC (33, 34). Gabriels et al. found that inducing vascular stress was sufficient to upregulate Cx43 expression in rat aorta EC, indicating a role for Cx43 in the EC stress response (31). In this study, immunofluorescent imaging detected colocalized Cx43 and Cx40 signals in aortic branch EC, while Cx37 was notably absent from Cx43 expressing regions (31). These results suggest that Cx43 expression is dynamic and regulated depending on factors including sheer stress to maintain the function of large artery EC.

Cx43 in conduit vessel vSMC

Cx43 is robustly expressed in large artery vSMC of multiple species (**Figure 1**), including in the human, rat, mouse, rabbit, bovine coronary arteries, aorta, and carotids (26, 32, 33, 36-40). In mouse carotid arteries, Cx43 appears as small punctates at the membrane of vSMC, suggesting gap junction formation. Despite being readily identified, reports have suggested that only minimal gap junction transfer occurs in vSMC (41). Cx43 expression also differs by vessel location. In the rat, greater levels of Cx43 are present within vSMC of the ascending and arch regions of the aorta, while decreased Cx43 levels are found in the descending aorta (42). Taken together, these data suggest that only limited Cx43 gap junction communication occurs in vSMC of conduit vessels in physiological states. This likely reflects vSMC function in these vessels, providing support to the vessel wall against cardiac output pressures rather than coordinating contractile responses as occurs in resistance arteries.

Cx43 in atherosclerosis

Atherosclerosis is a disease affecting the conduit vasculature characterized by a build-up of cholesterol plaques within the subintimal space. These plaques occlude blood vessels and limit blood flow leading to localized tissue ischemia. In human coronary artery vSMC, Cx43 expression is elevated in the early stages of atherosclerosis compared with healthy arteries (40). Increases in Cx43 expression are also noted in EC within the shoulder region of advanced atheromas that experience inherent increases in turbulent flow (43). Coincidentally, EC Cx37 and 40 are no longer detectable in these areas of Cx43 upregulation, suggesting compensation between the different connexin isoforms (43). In human late-stage atherosclerosis, vSMC Cx43 expression decreases to levels below that of healthy arteries (40). Similar decreases in Cx43 expression have been observed in mouse and rabbit models of atherosclerosis at the mRNA and protein level, suggesting a time-dependent role of Cx43 in atherosclerotic disease stages (38, 43). However, there is still limited understanding of the role of Cx43 or gap junction signaling in disease progression.

Modulating Cx43 can influence multiple aspects of atherosclerotic plaque development. For example, in $LDLR^{-/-}$ mice prone to atherosclerotic lesions, Cx43 heterozygous knockdown ($Cx43^{+/-}$ mice) results in reduced formation of atherosclerotic lesions compared to $Cx43^{+/+}$ $LDLR^{-/-}$ mice (44, 45). Additionally, atherosclerotic plaques present in $LDLR^{-/-}$ $Cx43^{+/-}$ mice show indications of increased stability, reduced inflammatory cells, and a thicker fibrous cap (45). HMG-CoA reductase inhibitors, or statins, are prescribed to patients because they are known to reduce the risk of plaque rupture. Administration of HMG-CoA reductase inhibitors to hypercholesterolemic

mice results in a reduction in the expression of Cx43, suggesting that Cx43 may decrease plaque stability (44). Morel et al. investigated the role of Cx43 in vascular inflammation and demonstrated reduced plaque formation and neutrophil recruitment in $LDLR^{-/-}$ mice with $Cx43^{+/-}$ bone-derived macrophages compared to $Cx43^{-/-}$ and $Cx43^{+/+}$ macrophages, indicating a role for Cx43 gap junction signaling in immune cell-mediated plaque development (46). Although the beneficial effect of Cx43 reduction was not seen in the $Cx43^{-/-}$ mice, the authors suggested this may result from compensatory mechanisms involving other connexins (46). In vSMC, posttranslational modifications of Cx43 may also influence atherosclerotic disease states by regulating vSMC proliferation (37). Atherosclerosis-associated oxidized phospholipid derivative 1-palmitoyl-2-oxovaleroyl-sn-glycerol-3-phosphorylcholine (POVPC) exposure in mouse carotids altered in vSMC phenotype leading to increased vSMC proliferation, which was associated with increased mitogen-activated protein kinase (MAPK) Cx43 phosphorylation at Cx43-S279/282 (37). However, another oxidized phospholipid derivative which does not induce atherosclerosis, 1-palmitoyl-2-glutaroyl-sn-glycerol-3-phosphorylcholine (PGPC) increased Cx43-S368 phosphorylation by protein kinase C pathways, which was not associated with increased vSMC proliferation (37). These studies suggest Cx43 and gap junction signaling is involved in regulating atherosclerotic plaque development and that the specific phosphorylation state of Cx43 may play a role in regulating cell functions during disease development. However, it is still unclear what role this plays in disease progression.

Cx43 in restenosis/neointima formation

Interventional treatment of conduit artery atherosclerosis involves angioplasty and stent implantation to restore the artery's luminal diameter. Placement of stents can damage the blood vessel wall, stripping endothelium and exposing the internal elastic lamina. These changes can trigger restenosis at the site of intervention. Restenosis is caused by vSMC proliferation, leading to medial expansion and neointima formation, which narrows the blood vessel lumen (47). This results in around a 6-year 17% late stent failure rate, regardless of the use of drug-eluting stents (48). Following balloon injury in rat carotid arteries, Cx43 is upregulated in medial vSMC and in the forming neointima up to 14 days (39). However, in rabbits subjected to balloon iliac artery injury, Cx43 mRNA expression in vSMC is unaltered compared to control animals when examined four weeks post-operatively (38). These data suggested that Cx43 may play a role in controlling vSMC proliferation during the initial stages of neointima formation.

Although Cx43 has been reported to influence neointima development, its specific role is still poorly understood. Chadjichristos et al. used a high-fat diet in mice with a global reduction of Cx43 expression ($Cx43^{+/-}$ $LDLR^{-/-}$) and found reduced vSMC infiltration and proliferation following carotid balloon injury compared to $Cx43^{+/+}$ $LDLR^{-/-}$ control littermates (49). These data suggest that reducing the expression of Cx43 gap junctions may limit neointima formation after endothelial injury. However, these findings appear to be in direct contrast with the results obtained by Laio et al. (50). In their studies, vSMC-specific Cx43 knockout showed significantly increased neointima formation following damage to arterial endothelium by either wire injury or carotid ligation (50). The opposing findings between these studies may be attributed to experimental variation in diet, adaptive vascular processes, type of endothelial injury, and connexin-mediated intercellular communications.

The specific role of gap junctions in neointima formation has not been well established. Studies blocking Cx43 channels with carbenoxolone and peptide Cx43 channel blocker, Gap26, reduced neointima formation after vascular injury in rats, suggesting a gap junction channel role in neointima development (51). Johnstone et al. also demonstrated non-gap junction effects of Cx43 in neointima formation (26). In this study, carotid ligation injuries increase vSMC MAPK-Cx43 phosphorylation associated with vSMC proliferation and neointima formation, which was lost in knock-in mice containing global alanine substitutions at MAPK-Cx43 serines (26). These changes were suggested to occur independent of gap junction signaling and were linked to Cx43 binding of known cell-cycle promoters, cyclin E, and CDK2 to promote neointimal formation (26). Given the complex nature of the disease and conflicting data, it is possible that both gap junction signaling and connexin-mediated protein interactions contribute to neointima formation, with multiple cell types interacting to control disease progression. More research is needed to fully elucidate the mechanism by which Cx43 regulates vSMC injury response in neointima formation.

Cx43 in the contractile vasculature

Resistance arteries and smaller arterioles are primarily responsible for regulating blood pressure and vascular resistance due to these vessels' muscular/contractile nature. Regulation of blood vessel tone occurs through communication between homologous cell types (homocellular signaling) and between different cell types (heterocellular signaling) (52-55). Cx43 containing gap junctions are present in contractile vessels (**Figure 1**). They contribute to the propagation and regulation of vasoconstricting and vasodilatory signals by facilitating direct homocellular and heterocellular coupling of EC and vSMC (56-58).

In 1986 Segal et al. demonstrated that vasodilator acetylcholine propagates and disperses vasodilation both with and against the direction of blood flow. This finding implied the role of direct cell-to-cell coupling in facilitating the movement of vasoregulatory signals, termed conducted vasomotion (59). Dye tracing studies show that direct coupling between vascular cell types results from gap junction formation (41). These gap junctions facilitate electrical conduction and the movement of signaling molecules across the endothelium and to the neighboring vSMC (60, 61). Gap junctions containing Cx43 regulate vSMC Ca^{2+} concentration often by the exchange of signaling molecules, including inositol 1,4,5-trisphosphate (IP_3), which facilitates the release of Ca^{2+} from intracellular stores (11, 62). This increase in cytoplasmic vSMC Ca^{2+} results in cell contractility (62). There is also evidence that Cx43 containing gap junctions are involved in vasodilatory processes such as endothelial-derived hyperpolarization (EDH), which is discussed in detail in the following section on Cx43 at the myoendothelial junction (63-68).

Resistance vessel EC Cx43

Cx43, Cx40, and Cx37 gap junctions have all been identified in the EC of the contractile vasculature and are thought to contribute to vasomotor responses (8, 69, 70). Cx43 couples EC across contractile vessel beds (**Figure 1**). Cx43 is in the EC of mouse cremaster arterioles, although to a lesser extent than Cx37 and Cx40 (71). Little et al. detected Cx43 in rat cremaster, rat brain, and hamster cheek pouch arteriole EC (8), and Gustafsson et al. identified Cx43 in rat mesenteric arteriole EC and in rat mesenteric resistance artery EC using immunofluorescence (70). In their studies, Cx43 containing gap junction plaques in EC were larger and more abundant than the Cx43 plaques in medial vSMC layers of the vessels (8, 70).

Cx43 hemichannels are also present in EC, where they function as purinergic release channels (14, 15, 72). Purinergic ATP release is associated with immune responses, including neutrophil activation/infiltration and regulation of vascular tone by facilitating NO/prostacyclin release resulting in vasodilation (73, 74). Multiple studies have demonstrated that blocking hemichannel function attenuates ATP release in cultured human microvascular EC (15, 75). Hemichannel blocking connexin mimetic peptide JM2 also reduced vascular inflammation in vivo in a rat model of silicone device implantation (75). The Pannexin 1 (Panx1) proteins form single membrane channels with similar functions to connexin hemichannels in EC and are known to act as purinergic release channels, which has led to some potential discrepancies in the literature as to the role of hemichannels versus pannexin channels. Studies by Lohman et al. showed that depletion of Cx43 at the cell membrane of cultured EC did not significantly reduce membrane

Panx1 and did not affect the ability of cells to release ATP (76). These findings suggest Panx1 channels may play a more significant role than Cx43 in purinergic ATP release (76). Multiple studies have used different methods to differentiate connexin and pannexin functions. However, a lack of channel-specific inhibitors and a limited understanding of the specificity of connexin mimetic peptides has further complicated the process of studying the role of these channels individually, as we recently reviewed (77).

Resistance vessel vSMC Cx43

Early investigations identified Cx43 in vSMC of rat brain and cremaster microvessels and hamster cheek pouch arterioles (8). Multiple other studies failed to identify Cx43 in rat mesenteric arteriole vSMC (70, 78). At the same time, Matchkov et al. identified discrete Cx43 plaques in vSMC of the rat superior mesenteric artery but not in smaller third-order mesenteric vessels (79). In contrast, Wang et al. reported elevated protein expression of Cx43 in whole third-order rat mesenteric arterioles compared to first-order arterioles by western blot (80). However, it should be noted that these findings were not specific to vSMC, given the techniques used (80).

The degree of Cx43 mediated vSMC homocellular coupling across species, and tissue beds has not been fully elucidated. In 1992, Christ et al. showed that the delivery of Ca^{2+} to cultured Cx43 positive SMC, derived from human corpora cavernosa, increased Ca^{2+} levels of adjacent cells, implying the direct movement of Ca^{2+} from one cell to another through Cx43 gap junctions (62). Jiang et al. published electrophysiological evidence of vSMC coupling by Cx43 and Cx40 in rat basilar arteries (81). This study identified single gap junction channels containing Cx43 and Cx40 (81). The composition of these channels can influence cell-to-cell conductance (81). Borysova et al. tested the role of gap junctions in Ca^{2+} signaling and vasoconstriction in isolated rat mesenteric resistance arteries (82). The non-specific gap junction inhibiter 18β-glycyrrhetinic acid prevented the spread of Ca^{2+} spikes between vSMC when the endothelial layer was denuded (82). Cx43 was specifically implicated in coordinating vSMC-to-vSMC contraction in rat mesenteric arteries when Cx43 mimetic peptide and channel blocker, ^{43}Gap26, attenuated arginine vasopressin-induced, endothelium-independent, vasoconstriction (83). Even so, reports of direct coupling between vSMC are variable. For example, Haddock et al. showed that endothelial denudation in the primary branch of rat basilar arteries attenuated Ca^{2+} synchronization in vSMC (84). Little et al. found limited lucifer yellow dye transfer between vSMC compared to EC, suggesting altered channel composition, number, or regulation between EC and vSMC (41).

Cx43 at the myoendothelial junction

In the contractile vasculature, MEJ gap junctions facilitate EC to vSMC communication and vice versa (41). MEJ form by direct contact between EC and vSMC through breaks in the internal elastic lamina (85). These structures are visible primarily by electron microscopy (86) but have also been identified in vessels using confocal microscopy (58). Studies in rat models identified a primarily EC projection origin for MEJ formation (87), while studies in humans revealed MEJ originating from vSMC projections 40% of the time (88). The presence of Cx40 and Cx43 at the MEJ was confirmed in vivo by immunofluorescent imaging of mouse cremaster arterioles (58). In rat basilar arteries, Cx37 and Cx40 are the only connexins found at the MEJ (84). These results suggest that the Cx43 contribution to MEJ signaling may vary between species or between vascular beds (84). MEJ-mediated heterocellular signaling has also been demonstrated in EC and vSMC co-culture models (13, 27, 89). Both Cx40 and Cx43 are the dominant gap junction forming proteins expressed in endothelial MEJ *in vitro*, while only Cx43 was found at vSMC MEJ (89).

Cx43 gap junctional activity at the MEJ is tightly regulated to ensure appropriate vascular tone. For example, cyclic AMP (cAMP) promotes Cx43 gap junctional permeability, assembly, and protein kinase A phosphorylation, and as a consequence, its presence at the MEJ regulates EC and vSMC communication (90-93). Xu et al. identified increased cAMP levels in rat EC and vSMC co-cultures in hypoxic conditions (13). These increases in cAMP correlate with increased Cx43 expression at the MEJ (13). In the same study, cAMP also passed through Cx43 containing MEJ gap junctions, increasing vSMC cAMP levels (13). cAMP-mediated hyperpolarizing effects and modulation of vSMC, as well as movement through Cx43 channels, are necessary for the regulation of vascular tone (94). Additionally, cAMP-mediated alteration of Cx43 phosphorylation at the MEJ increases gap junction permeability and intracellular communication either through a reduction in Cx43 PKC phosphorylation (known to close channels) or an increase in protein kinase A phosphorylation (known to open channels) (13, 27).

In contractile vasculature, EDH results in vSMC hyperpolarization followed by vessel relaxation. EDH occurs when Ca^{2+}-induced opening of IK_{Ca} and/or SK_{Ca} channels and subsequent efflux of K+ hyperpolarizes EC, where the signal spreads to vSMC (95). Multiple studies have implicated gap junctions at the MEJ in coordinating EDH responses (64, 96, 97). In resistance arteries obtained from subcutaneous fat biopsies in pregnant women, Cx43 was identified as the most significant connexin for human vessel EDH (98). To test the role of specific connexins, including Cx43, in spreading hyperpolarization, studies aimed to block other vasodilatory signaling

pathways, including nitric oxide (NO) as well as prostaglandin signaling (63-68). These studies then incorporated approaches to disrupt gap junctional communication (63-68). One routine method is the use of connexin mimetic peptides to disrupt connexin function. We have previously reviewed the formulation, specificity, and use of these peptides (77). Peptides of consensus sequences to Cx43 were designed to bind to Cx43 regions and limit function by closing channels. However, the specific binding of most connexin mimetic peptides to channels has not been demonstrated (77). Application of a Cx43 mimetic peptide and gap junction blocker Gap27 attenuated EDH responses across multiple studies, indicating that Cx43 contributes to spreading hyperpolarization signals from EC to vSMC (63-67). It is important to note the potential lack of connexin specificity and cross-reactivity of Gap27 blockers, which have been shown to block other connexin channels, including Cx40, and pannexin channels, as we recently reviewed (77).

Cx43 containing gap junctions can also regulate blood vessel tone by facilitating signaling from vSMC to EC. In 1997 Dora et al. observed that increases in vSMC intracellular Ca^{2+} cycling following the application of vasoconstrictors phenylephrine and KCl could result in a subsequent rise in EC intracellular Ca^{2+} (54). Ultimately this led to endothelial-specific increases in NO production, resulting in vSMC relaxation (54). The authors hypothesized that gap junctions present at the MEJ facilitate the movement of Ca^{2+} from vSMC to EC, elevating NO levels in EC (54). In 2005 Lamboley et al. discovered that inhibiting IP_3 production in response to elevated Ca^{2+} levels in vSMC prevented a rise in EC Ca^{2+} concentrations (99). This finding implied the involvement of MEJ gap junctions in the movement of IP_3 from vSMC to EC and subsequent increases in IP_3-induced elevation of Ca^{2+} in EC (99). Later studies confirm that gap junction inhibitors prevent rises in vSMC Ca^{2+} from causing subsequent increases in EC Ca^{2+} (100, 101). This signaling across the MEJ likely occurs through channels composed of Cx43, Cx37, and Cx40 (100).

Cx43 gap junctions are post-translationally regulated, including at the MEJ. These post-translational modifications are often associated with changes in channel function and may be a mechanism by which cells regulate EC and vSMC communication. Serine Cx43-S368 phosphorylation is present in mouse cremaster arterioles at the MEJ (58). Cx43-S368 phosphorylation is associated with decreased channel opening and reduced signaling between EC and vSMC at the MEJ in cells isolated from mouse contractile vessels (27, 102). Additionally, post-translational modifications like S-nitrosylation at Cx43-C271 can increase Cx43 channel permeability. Straub et al. found an enrichment of active eNOS in mouse first order thoracodorsal arteries and an increase in Cx43-C271 S-nitrosylation (28). This finding corresponded with an

increase in the movement of IP_3 across the MEJ, indicating an increase in Cx43 channel permeability (28). S-nitrosoglutathione reductase, an enzyme known for promoting denitrosylation, was also identified at the MEJ following the application of phenylephrine, a vasoconstrictor (28). Taken together, this evidence suggests that posttranslational modifications play a significant role in regulating Cx43 gap junction permeability and coordination of vascular heterocellular signaling at the MEJ and vessel tone.

Cx43 and hypertension

Cx43 plays an essential role in regulating normal contractile function in the resistance vasculature, and as such, alterations in Cx43 expression can influence or be influenced by resistance vessel dysfunctions, including hypertension. In contractile arteries, the expression of vascular connexins, including Cx43, are altered in hypertension. In mouse mesenteric vSMC, renin-dependent hypertension is associated with an angiotensin II-induced upregulation of Cx43, while renin-dependent and independent hypertension increased EC Cx40 and SMC Cx37 and Cx45 (103). Similarly, spontaneously hypertensive rats showed increased Cx43 expression in mesenteric vascular vSMC (80). In these hypertensive rats, the delivery of a gap junction blocker, niflumic acid, produced less vasorelaxation compared with normotensive controls. Niflumic acid also reduced Cx43 expression resulting in a decrease in blood pressure (80). Other studies using hypertensive animal models demonstrated that gap junctional activity between vSMC was increased in conjunction with elevated Cx45 expression and alterations in Cx43 phosphorylation states (80, 104). Taken together, these studies suggest that increases in Cx43 expression and gap junctional communication contribute to a hypertensive state, potentially facilitated by increased vSMC homocellular coupling. EC-targeted Cx43 knockout mice were hypotensive and had elevated plasma NO levels (105). The authors hypothesized that these observations were due to reductions in EC Cx43 gap junction mediated cell-to-cell communication, leading to increased EC Ca^{2+} and ultimately increased NO production (105).

Cx43 in the capillary network

Capillaries are the smallest vessels ranging between 5-10 μM in diameter. Capillaries allow for the exchange of gas, water, nutrients, and waste between the blood, cells, and interstitial fluids. Capillary walls are made of EC attached to the basement membrane by integrins, without vSMC or internal elastic lamina. Pericytes are found within the vascular basement membrane, where

their cytoplasmic processes surround the abluminal surface of EC. The ratio of EC and pericytes vary depending on tissue function (106).

Cx43 in capillary EC

Cx43 is reported in capillary EC across species and tissue beds (**Figure 1**). Cx43 is present in mouse and human retinal blood vessels (107). In mouse retina whole mount tissues, Cx43 was detected specifically in the EC (108). Cx43 expression has also been demonstrated in mouse and rat alveolar capillary beds (16) and in renal peritubular capillaries of the cortex and outer medulla in rat and mouse (109). However, some studies have reported no Cx43 in rat glomerular capillaries (109) and mouse skeletal muscle capillaries (71). Cx43 expression has also been confirmed in mouse blood-brain barrier EC (110, 111). In studies of Ca^{2+} signaling, EC-specific Cx43 knockout and the use of peptide Cx43 gap junction inhibitors were associated with reduced Ca^{2+} spread along alveolar capillaries (16). This evidence suggests functional Cx43 gap junction formation between capillary EC in vivo (**Figure 1**).

Cultured microvascular EC derived from rat epididymal pads express Cx43, detected by western blot (112). Mouse brain microvascular EC express Cx43 in culture and in vivo (110, 111). Cx43 is also present in primary cultured human retinal microvascular EC (hRMEC) (107). Immunofluorescence staining identified the dense localization of Cx43 around hRMEC nuclei but at cell membranes in human retinal vessels, suggesting differences in levels of gap junctional connectivity between culture and animal models (107). It should be noted that differences in Cx43 expression in cultured cells may result from supplementation of media with growth factors altering the cellular environment.

Cx43 in pericytes

Pericytes extend processes around the outside of capillaries and are thought to regulate capillary blood flow and permeability in addition to being involved in angiogenesis (113). In the brain, pericytes are important for controlling cerebral blood flow, maintenance of the blood-brain barrier, and regulating inflammatory cell infiltration. Cx43 expression in pericytes has been reported by several model systems and at specific developmental time points. Still, various studies have questioned Cx43 as the predominant connexin isoform in all pericytes. Cx43 expression has been identified in human retinal pericytes, human brain vascular pericytes, and embryonic-stage pericyte precursor cells (114-117). Specifically, Cx43 punctates were localized to pericyte cell membranes when co-cultured with EC (115). In whole-mount mouse retina, small punctates of

Cx43 protein were identified between pericyte membranes (118). These data suggest the formation of gap junctions between pericytes and potentially between pericytes and EC (**Figure 1**). However, single-cell RNA sequencing studies have questioned capillary Cx43 expression in various tissues, including mouse brain, heart, and skeletal muscle (119, 120).

Cx43 capillary gap junctions

Junctional transfer of small molecules between EC and pericytes was described as early as 1987 by Larson et al. The co-culture of microvascular EC and pericytes from the bovine brain showed the transfer of lucifer yellow dye and nucleotides (121). The transfer of cascade blue between Flk-1-eGFP+ ECs and NG2-DsRed+ PC/precursors has been demonstrated by Payne et al. in mouse embryonic stem cells (117). Cx43 gap junctions are reported to allow for communication between pericytes and EC in brain tissue and retinal capillaries (122, 123). Dye transfer studies using the tracer neurobiotin indicated that gap junctions form between pericytes and propagate signals in the retina microvasculature (122). Pericyte precursor cells in embryonic endothelium were found to be coupled by Cx43 containing gap junctions during vascular assembly, suggesting that Cx43 is involved in vascular development. Cx43 knockout in $Ng2^{+}$ pericyte precursor cells leads to abnormal vessel development in early embryos, although adult retinal vessels appeared relatively normal (117). This may suggest a compensatory role by other connexin isoforms (117).

Cx43 and EC permeability

Regulation of EC barrier function is essential to maintaining appropriate exchange between vessels and tissues, although the precise role of Cx43 in EC permeability has not been fully elucidated. In cultured porcine-derived EC from blood-brain barrier vessels, Cx43 was colocalized and coprecipitated with tight junction proteins: occludin, claudin-5, and zonula occludens-1 (ZO-1) (124). Thrombin-induced lung microvascular permeability was decreased in the presence of Cx43 gap junction inhibitors Gap26 and Gap27 in rat lungs, suggesting the role of gap junctional coupling in regulating EC barrier function (16). Gap27 also decreased EC permeability following acid-induced injury in the rat lung microvasculature (125). In contrast, gap junction blockers 18beta-glycyrrhetinic acid (18beta-GA) and oleamide impaired the tight junction barrier function of cultured primary porcine brain microvascular EC as measured by transendothelial electrical resistance. Still, they did not alter the expression or subcellular localization of connexins or tight junction proteins (124). Further studies in cultured rat retinal EC found that decreased expression

of Cx43 correlated with increased cell monolayer permeability and reduced expression of tight junction proteins ZO-1 and occludin (126). Although these studies were performed in cultured microvascular EC, it is important to note that normal EC permeability is regulated at the postcapillary venous level (127). These data suggest an essential role for Cx43 in maintaining tight junctions and capillary barrier function, although more studies are required to understand the mechanisms of control.

Angiogenesis

The migration and proliferation of endothelial progenitor cells in capillaries are essential features of angiogenesis (128, 129). Limited studies have defined a role for Cx43 in angiogenic responses. Studies by Mannell et al. revealed that Cx43 promotes EC migration and angiogenesis through tyrosine phosphatase SHP-2 mediated pathways (130). The knockdown of Cx43 by lentivirus transduction of siRNA, or mutant SHP2 in EC, decreased EC migration rates (130). In vivo and in vitro mouse studies also suggest that Cx43 promotes EC-mediated angiogenesis by activating protein kinase A signaling and upregulating the expression of hypoxia-inducible factor-1α (HIF-1α) as well as vascular endothelial growth factor (VEGF) (131).

Cx43 in diabetes

Disruptions in the capillary network are characteristic of diabetic disease and can result in retinopathy and nephropathy (132). In vitro culture of human retinal EC and pericytes exposed to high glucose levels showed a reduction in Cx43 expression and impaired gap junction activity (112, 114). In cultured rat retinal EC, Cx43 expression and gap junction intercellular communication was significantly reduced by high glucose treatment (126). Both high glucose or Cx43 knockdown by siRNA reduced the protein expression of occludin and tight junction protein (ZO-1), resulting in increased cell permeability of cultured rat EC (126). These findings suggest a link between decreased Cx43 expression, decreased tight junction protein expression, and increased retinal capillary permeability in diabetes. Kim et al. reported high glucose conditions lead to an increase in Rab20, a protein thought to regulate intracellular Cx43 trafficking (133). Increases in Rab20 were associated with impaired gap junction-mediated signaling and decreased Cx43 plaque number (133). Reducing Rab20 expression by siRNA approaches restored the expression of Cx43, improved gap junctional intercellular communication, and reduced high glucose-mediated cell apoptosis (133).

In a streptozotocin-induced mouse model of diabetes, expression of Cx43 was reduced in retinal tissue, accompanied by pericytes loss (134). In addition, pericyte-mediated neurobiotin transfer was significantly reduced in streptozotocin-induced diabetic rat retinal capillaries, suggesting a reduction in gap junctional coupling after the onset of diabetes (122). Lentiviral-induced knockdown of Cx43 in diabetic rats led to increased apoptosis of retinal vascular cells, pericyte loss, vascular leakage, and the formation of acellular capillaries, or capillaries that lack nuclei along their length (21). Cx43 expression was reduced in human diabetic patient retinas, associated with increased vascular cell death (135). On the contrary, the Akimba mouse model of advanced proliferative diabetic retinopathy exhibited increased retinal Cx43 expression (107). Cx43 expression was also increased in vitro in primary retinal microvascular EC and human donor tissues from patients with proliferative diabetic retinopathy (107). These findings contradict those from other studies of diabetic retinas (134, 135), potentially because of the differences between models, the severity of disease, and vessel location.

Conclusion

Although the expression of Cx43 varies by blood vessel size, location, cell type, and species, it is clear that Cx43 plays an essential role in maintaining healthy blood vessel function. This includes regulating homocellular and heterocellular signaling between vSMC, EC, and pericytes in the vessel wall. Cx43 contributes to the maintenance of vascular tone, proliferation, angiogenesis, and EC barrier function. The necessity of Cx43 is highlighted by the fact that changes in Cx43 expression, localization, and posttranslational modification are often observed in vascular disease states, including atherosclerosis, hypertension, and diabetes. However, more research is required to understand the exact nature of Cx43 gap junctions, hemichannels, and protein interactions.

Developments in the field promise to provide advanced tools for studying connexins. One such tool is rationally designed connexin mimetic peptides intended to modulate Cx43 gap junction activity and protein-protein interactions (92). Since their development, these peptides have been used to achieve inhibition of specific gap junction channels, yet the selectivity of these channel blockers is in question (77). Non-gap junctional roles for Cx43 is an emerging area of investigation. Several connexin mimetic peptides have been developed to target these functions in cancer and cardiovascular disease (26, 136-139). Mimetic peptide development is a promising strategy for the treatment of vascular disease as these peptides can modulate connexin phosphorylation states as well as protein-protein interactions (26, 136-139). Although much work has been done on the role of Cx43 in the vasculature, more work is required to understand the contribution of gap junctions and connexins in physiology and the pathology of vascular diseases.

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Conflict of Interest Statement
The authors have no conflicts of interest to declare.

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Figure 1: Cx43 localization in conduit arteries, resistance arteries, and capillaries. Cx43 is present in vSMC of conduit and resistance arteries. In conduit arteries at branch regions and areas of turbulent flow and high shear stress, Cx43 expression is found in EC as well as SMC. In contractile (resistance) arteries Cx43 is expressed in VSMC and EC and localizes to the MEJ where it couples EC and vSMC. In capillaries, Cx43 is expressed in EC and pericytes and permits EC-pericyte signaling.