Cx37 deletion enhances vascular growth and facilitates ischemic limb recovery

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Fang JS, Angelov SN, Simon AM, Burt JM. Cx37 deletion enhances vascular growth and facilitates ischemic limb recovery. Am J Physiol Heart Circ Physiol 301: H1872–H1881, 2011. First published August 19, 2011; doi:10.1152/ajpheart.00683.2011.—The unique contributions of connexin (Cx)37 and Cx40, gap junction-forming proteins that are coexpressed in vascular endothelium, to the recovery of tissues from ischemic injury are unknown. We recently reported that Cx37-deficient (Cx37−/−) animals recovered ischemic hindlimb function more quickly and to a greater extent than wild-type (WT) or Cx40−/− animals, suggesting that Cx37 limits recovery in the WT animal. Here, we tested the hypothesis that enhanced angiogenesis, arteriogenesis, and vasculogenesis contribute to improved posts ischemic hindlimb recovery in Cx37−/− animals. Ischemia was induced unilaterally in the hindlimbs of WT or Cx37−/− mice (isoflurane anesthesia). Postsurgical limb appearance, use, and perfusion were documented during recovery, and the number (and size) of large and small vessels was determined. Native collateral number, predominantly established during embryonic development (vasculogenesis), was also determined in the pial circulation. Both microvascular density in the gastrocnemius of the ischemic limb (an angiogenic field) and the number and tortuosity of larger vessels in the gracilis vasculature (an arteriogenic field) were increased in Cx37−/− animals compared with WT animals. Cx37−/− mice also had an increased (vs. WT) number of collateral vessels in the pial circulation. These findings suggest that in Cx37−/− animals, improved recovery of the ischemic hindlimb involves enhanced vasculogenesis, resulting in increased numbers of collaterals in the hindlimb (and pial circulations) and more extensive collateral remodeling and angiogenesis. These results are consistent with Cx37 exerting a growth-suppressive effect in the vasculature that limits embryonic vasculogenesis as well as arteriogenic and angiogenic responses to ischemic injury in the adult animal.

gap junction; connexin; ischemia; collateralization; angiogenesis; arteriogenesis

GAP JUNCTIONS are plaques of intercellular channels that mediate the passage of electrical and chemical signals. More than 20 connexin (Cx)-encoding genes have been identified in the human genome (32). Each isoform is expressed in a tissue-specific manner and forms channels that differ in their permeability and gating characteristics. Distinct primary sequences of their cytoplasmic regulatory domains suggest that different Cx isoforms may play isoform-specific, if partially overlapping, regulatory roles even if coexpressed. In the vasculature, four Cx isoforms (Cx37, Cx40, Cx43, and Cx45) are commonly expressed, with Cx37 and Cx40 coexpressed and predominant in the endothelium. Recently, we reported that Cx37-deficient (Cx37−/−) animals recovered limb perfusion, appearance, and use better than wild-type (WT) or Cx40−/− animals after challenge with severe hindlimb ischemia (5). Critical to the survival and recovery of ischemic tissues are several mechanisms that acutely or chronically improve blood flow downstream of hypoxic tissue, including ischemia-induced vasodilation of upstream sites via conducted vasomotor responses, flow-mediated remodeling of upstream collateral vessels, and microvascular angiogenesis. Which of these essential recovery mechanisms might be enhanced in Cx37−/− animals to improve posts ischemic limb health and function remains unexplored.

Improved vasodilatory capacity at upstream feed arteries and collaterals, and enhanced conduction of vasodilatory signals along the vascular endothelium, would be predicted to enhance recovery of the ischemic hindlimb. However, available evidence does not suggest that these processes would be enhanced in Cx37−/− animals. Cx37 has been shown to bind to, and colocalize with, endothelial nitric oxide (NO) synthase (eNOS) (23); this protein-protein interaction might facilitate local and upstream vasodilation by activating eNOS activity to upregulate local NO production. However, NO donor application reduces electrical coupling of cultured microvascular endothelial cells (ECs) (19) and dye coupling (but not electrical coupling) of Cx37-expressing cultured nonvascular cells (13). In intact arterioles, upstream propagation of KCl-stimulated vasoconstriction is reduced in Cx37-deficient animals (20); however, conducted vasodilatory responses to locally applied ACh do not differ between Cx37-deficient and WT mice (7). Although these data indicate clear differences in vasomotor function between WT and Cx37−/− mice, they do not suggest that the profound improvement in the recovery of posts ischemic hindlimb blood flow and use observed in the Cx37−/− mouse (5) can be fully explained by enhanced local vasodilation (in response to ischemia) or vasodilatory conduction to upstream sites.

Enhanced vascular remodeling capacity would also be predicted to enhance recovery of ischemic limb function. Despite the characteristic low cellular turnover of ECs (16), physiological and pathological changes in growth factor availability, flow profile, or downstream O2 content stimulate several vascular growth mechanisms, including angiogenesis (growth of new microvessels, generally sprouting from existing vessels), arteriogenesis (remodeling of existing vessels to increase lumen diameter), and possibly vasculogenesis (de novo growth of blood vessels, typically restricted to developmental periods); these vascular growth and remodeling processes are, in turn, capable of extending the existing vasculature to enhance downstream perfusion. Vascular remodeling involves the activation of growth and proliferation responses in cells of the vascular wall and relies on the appropriate integration of a diverse array of injury-related signaling molecules. Despite ample evidence supporting growth regulatory roles for gap junctions and their constituent connexins (14), few studies have specifically ad-

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Table 1. Characteristics of experimental animal groups in which unilateral hindlimb ischemia was induced by the indicated models and recovery of limb appearance and use were assessed

<table>
<thead>
<tr>
<th></th>
<th>Number of Animals</th>
<th>Percent Male</th>
<th>Mean Initial Animal Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>FSAVPR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>13</td>
<td>54</td>
<td>21.4 ± 0.8</td>
</tr>
<tr>
<td>Cx37−/−</td>
<td>13</td>
<td>54</td>
<td>22.1 ± 1.7</td>
</tr>
<tr>
<td>FAL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>12</td>
<td>67</td>
<td>20.5 ± 0.8</td>
</tr>
<tr>
<td>Cx37−/−</td>
<td>11</td>
<td>55</td>
<td>22.2 ± 1.2</td>
</tr>
</tbody>
</table>

FSAVPR, femoral-saphenous artery-vein pair resection; WT, wild-type; Cx37−/−, connexin37-deficient; FAL, femoral artery ligation.

dressed the involvement of vascular Cxs in vessel growth, remodeling, and repair responses, particularly in the context of ischemia. Cx37 is the only endothelial Cx to be downregulated in large vessels subjected to turbulent flow (8), where endothelial proliferation rates are upregulated (16). Abnormalities of vascular structure have not been reported for either Cx37−/− or Cx40−/− mice; however, vascular dysmorphogenesis and congestion are widespread in Cx37−/−/Cx40−/− mice, which die perinatally, likely from compromised lung function due to hemorrhage (28). The increased incidence of hemangiomas in the testes and intestine of Cx37−/− animals (28) is of particular interest because hemangiomas are typically associated with increased endothelial proliferation. These results suggest that Cx37 (and to some extent Cx40) may be essential to normal vascular development and responses to injury. Recently, we (1) reported that the expression of Cx37, but not Cx40 or Cx43, in highly proliferative rat insulinoma (Rin) cells arrests the progression of these cells through the cycle cells, slowing the cell cycle time from 2 to 9 days. In addition, Cx37 expression confers to Rin cells sensitivity to serum deprivation: Cx37-expressing Rin cells exposed to low serum conditions accumulated in the G1 phase of the cell cycle, indicating that Cx37 may suppress cell growth. Together, these reports suggest that growth inhibition by Cx37 may be a unique property of this protein compared with other vascular Cxs.

In the present study, we tested the hypothesis that enhanced vascular growth in Cx37−/− mice contributes to the improved recovery of ischemic limb perfusion and function in these animals. Our results support our hypothesis and suggest that, indeed, Cx37 serves a growth-suppressive role in the developing vasculature as well as in ischemia-induced vascular remodeling.

MATERIALS AND METHODS

Animals. In this study, 3- to 6-mo-old WT and Cx37−/− C57Bl/6 mice were used (27). Care was taken to ensure that comparable ratios of male and female animals were studied, and no differences in initial animal weights could be detected across groups (Table 1). Animals were permitted access to food and water ad libitum and were housed together when possible. All surgical procedures were performed under isoflurane in O2, and their hindquarters were depileated before placement on a heated Plaster of Paris block. Laser-Doppler scans were collected in triplicate using a Periflux Pim II (Perimed) with the threshold set at 5.6–5.7 V, values chosen based on scans taken of a dead animal, wherein flow is absent, and set for live scans to minimize background artifacts. Mean limb perfusion (in %) was calculated as the mean pixel intensity across three scans measured in an 11 × 24-pixel region of interest (ROI) encompassing the surgical paw and normalized for the same measurement taken of the control paw in each scan. The perfused area was measured as the number of pixels within each surgical limb ROI wherein flow was detected above threshold, again normalized to the control limb within each scan. Total limb flow (in %) was calculated as the comparison of surgical mean limb perfusion × perfused area against the control limb and again averaged across three scans for each time point.

Limb recovery. After the induction of hindlimb ischemia by either the FSAVPR or FAL models, surgical limb appearance and use were scored according to the parameters described in Table 1 by an investigator blinded to mouse genotype and surgical manipulation. Any animals experiencing severe necrosis or auto-amputation of distal

Table 2. Descriptors for the qualitative assessment of surgical limb appearance and use after the induction of ischemia by FSAVPR or FAL

<table>
<thead>
<tr>
<th>Score</th>
<th>Hindlimb Appearance</th>
<th>Hindlimb Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No difference in coloring (vs. the contralateral control limb)</td>
<td>Plantar flexion, toes respond to mild traction on the tail</td>
</tr>
<tr>
<td>−1</td>
<td>Mild redness (vs. the contralateral control limb)</td>
<td>Plantar flexion, no toe response to mild traction on the tail</td>
</tr>
<tr>
<td>−2</td>
<td>Moderate redness (vs. the contralateral control limb)</td>
<td>None or impaired plantar flexion; foot used to brace or balance</td>
</tr>
<tr>
<td>−3</td>
<td>Dark red or purple (vs. the contralateral control limb) or mild necrosis or sores</td>
<td>No plantar flexion; movement in hip during ambulation</td>
</tr>
<tr>
<td>−4</td>
<td>Severe necrosis or auto-amputation of distal tissue</td>
<td>Drugging of the hindlimb</td>
</tr>
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tissue in the surgical limb were prematurely killed, and subsequent hindlimb appearance scores were assigned a score of −4.

**Histology.** The gastrocnemius muscle was dehydrated by progressive alcohol washes and cleared with 100% xylene before being embedded in paraaffin. Cross-sections (7 µm thick) of each sample were mounted on glass slides, deparaffinized in xylene, and rehydrated by a progressive alcohol wash. Slides were blocked with 5% H2O2 (no. 216763, Sigma) in Ca2+-free PBS before an incubation with biotinylated Griffonia simplicifolia I (GS-1) lectin (1:200) (no. B-1105, Vector), a mixture of A and B group lectins that preferentially bind to α-N-acetylgalactosyl and α-galactosyl glycoproteins expressed on the surfaces of skeletal muscle capillary ECs (15). Binding of GS-1 lectin was revealed by treatment of incubated tissue with ready-to-use horseradish peroxidase-conjugated streptavidin (no. SA-5704, Vector) and DAB Chromogen substrate (no. K3466, DAKO), which generates an opaque substrate (see black spots in Fig. 2) at the site of positive lectin binding. Slides were counterstained with 1% methyl green (no. M5015, Sigma) in 0.1 N acetate buffer.

**Quantification of microvascular density and muscle fiber cross-sectional area.** Eight to twelve random fields were imaged for each lectin-treated section of control or surgical limb gastrocnemius tissue. Two analytical methods were used to calculate microvascular density. In method 1, ImageJ software was used to draw a 100-µm² ROI within each random field, and the number of lectin-positive particles was counted within that ROI to generate a microvascular density value (expressed in vessels/mm²). In method 2, ImageJ software was used to superimpose a grid of 16 × 22 intersects spaced 10 µm apart over each image, and the number of intersects coinciding with lectin-positive particles (Iₚ) or to the reference tissue (Iₑ) was counted for each field. The following stereological formula (18) was used to estimate the microvascular length density, the total capillary vessel sectional area.

\[ \text{vessel sectional area} = \frac{2 \times Iₚ \times a}{Iₑ \times \alpha} \]

where \( Iₚ \) is the microvascular length density, \( Iₑ \) is the total number of grid intersects and \( a \) is the actual area of the grid. In both methods, average microvascular density for each tissue was calculated as the mean value calculated across all images, and angiogenesis (in %) was calculated as the difference of surgical to control limb microvascular density. For the calculation of muscle fiber cross-sectional area, the areas of five randomly selected muscle fibers were measured in ImageJ software for each field and subsequently averaged across all images to obtain a mean value for each tissue.

**Hindlimb vascular fill.** Twenty-one days after the unilateral induction of hindlimb ischemia by FAL, animals were anesthetized by 1.5–5% isoflurane in O2, and FAL was performed on the contralateral Pial collateral fill. Animals were anesthetized by an overdose of 100 USP units heparin and were euthanized by an overdose of >100 mg/kg ketamine and >40 mg/kg xylazine administered intraperitoneally. The abdominal aorta was cannulated, and animals were perfused for 2 min with Ca2+-free PBS [containing 10−4 M sodium nitroprusside (no. S0501, Sigma) and 4 mg/l papaverin (no. P3510, Sigma)]. Animals were then perfusion fixed for 10 min with 1% paraformaldehyde in Ca2+-free PBS before perfusion of the vasculature with an 8:1 dilution of radioopaque microfill (MV-122, Flow-Tech) to fill the hindquarter vasculature. Hindlimbs were imaged by X-ray angiography (20-kV exposure for 10 s).

**Computed tomography.** Microcomputed tomography (micro-CT) images were collected with a Siemens Inveon micro-CT with the following setup: 401 projections were collected over 220°, 3 frames were summed per projection, and each frame was taken at 80 kV and 145-µA current (reconstructed pixel size of ~14 µm).

**Pial collateral fill.** Animals received an intramuscular injection of 100 USP units heparin and were euthanized by an intraperitoneal injection of >100 mg/kg ketamine and >40 mg/kg xylazine. The thoracic aorta was cannulated, and animals were perfused for 2 min with CaCl2-free PBS [containing 10−4 M sodium nitroprusside (no. S0501, Sigma) and 4 mg/l papaverin (no. P3510, Sigma)]. Animals were subsequently perfused with 5% Evans blue (no. E2129, Sigma) to mark the pial vasculature, and the intraparietal and parietal regions of the skull removed to expose the underlying pial vasculature. An 8:1 dilution of radioopaque yellow microfill (no. MV-122, Flow-Tech) to diluent was infused into the thoracic cannula and allowed to cast for 10 min. Brains were cleaned of extraneous tissue and fixed in 1% paraformaldehyde (in Ca2+-free PBS) overnight. Before being imaged, brains were counterstained by an immersion for 2 min in 2.5% cresyl violet (no. 101408, Merck) in distilled H2O and then washed in Ca2+-free PBS to remove extraneous dye. Regions of the brain were whole imaged at multiple focal planes on a stereomicroscope and manually reconstructed to optimize the visualization of individual vessels. For aesthetic purposes, whole images were color and contrast enhanced to match shades of purple and yellow across animals.

**Analysis of collateral characteristics.** After vascular fill of the hindquarters, X-ray angiograms of surgical and control limb gracilis muscles were analyzed using ImageJ software. A line was drawn perpendicular to the midpoint of the femur [line of interest (LOI)], and gracilis vessels that intersected this line were counted. Tortuosity ratio was measured by defining an upstream and downstream point on each vessel by 100 pixels from the LOI and comparing the actual point-to-point distance against the length of vessel connecting each point. By this ratio, a value of 1 indicates a nontortuous vessel, whereas a value >1 is indicative of vessel tortuosity. Vessel diameter was determined by measuring the width of each vessel at the intersection with the LOI and at the upstream and downstream points identified above. For the characterization of pial collaterals after cerebral vessel microfill, multiple images were taken of each brain hemisphere at multiple planes of focus and manually reconstructed to optimize vessel visualization. Collateral vessels connecting branches originating from the middle cerebral artery (MCA), posterior cerebral artery (PCA), and anterior cerebral artery (ACA) were identified and counted. Total counts were averaged for each brain hemisphere of each mouse to obtain mean pial collateral numbers.

**Statistics.** Nonparametric data (i.e., limb appearance and use scores) were compared using a Wilcoxon rank-sum test. All other data were compared using a Student’s t-test. In all tests, \( \alpha \) was set to 0.05.

**RESULTS AND DISCUSSION.** The purpose of the present study was to determine whether enhanced vascular growth in Cx37−/− animals could contribute to the improved recovery of hindlimb perfusion and function observed in this genotype after challenge with a severe ischemic insult by FASVPR. We (5) have previously reported, and confirm herein with a larger data set that includes sham surgery (sham)-treated animals (Fig. 1), that the hindlimbs of Cx37−/− animals subjected to FASVPR were not compromised in terms of appearance and use to the same extent as WT animals. Recovery of surgical limb appearance and use were assessed (qualitative descriptors shown in Table 2) at multiple postsurgical time points by an investigator blinded to animal genotype and surgical manipulation and statistically compared using a Wilcoxon rank-sum test for nonparametric data. As shown in Fig. 1, FASVPR significantly (vs. sham-treated animals) impaired surgical limb appearance and use in WT animals at most postsurgical time points, suggesting that a severe ischemic insult is induced in this model. Partial, but incomplete, recovery of limb use (but not limb appearance) was apparent in WT animals by day 14. In contrast, FASVPR had no impact on limb appearance (Fig. 1A) or use (Fig. 1B) in Cx37−/− animals; scores for these animals were statistically indistinguishable from sham-treated Cx37−/− animals and significantly improved relative to WT scores at most post-FASVPR time points.
Ischemic limb survival and recovery after FSAVPR involve the induction of angiogenesis and arteriogenesis to restore blood flow to hypoxic tissue distal to the site of ligation; augmentation of angiogenesis and arteriogenesis improves recovery (31), whereas deficiencies in these processes compromise recovery (4, 22, 26). Indeed, a sustained infusion of VEGF, a potent angiogenic activator, improves postischemic microvascular growth and angiogenesis. Furthermore, the enhanced postischemic recovery observed in Cx37/-/- mice might therefore result, in part, from an improved angiogenic capacity in these animals.

To determine whether angiogenesis is increased in the Cx37/-/- mouse, we visualized the microvasculature in cross-sections of gastrocnemius tissue harvested from the surgical and control limbs of WT and Cx37/-/- animals 14 days after the induction of ischemia by FSAVPR (Fig. 2A). Mean surgical limb microvascular density, assessed by counting the number of vessels in a 100-μm² ROI (method 1), was significantly elevated in FSAVPR-treated Cx37/-/- animals (1,474 ± 96 vessels/mm²) compared with FSAVPR-treated WT (1,204 ± 87 vessels/mm²) and sham-treated Cx37/-/- (1,086 ± 49 vessels/mm²).
vessels/mm²) controls (Fig. 2B). These values were compared against measures obtained for the contralateral control limbs of FSAVPR- and sham-treated Cx37⁻/⁻ animals (FSAVPR: 998 ± 59 vessels/mm² and sham: 1,051 ± 138 vessels/mm²) and WT animals (FSAVPR: 1,075 ± 51 vessels/mm² and sham: 1,212 ± 63 vessels/mm²) to calculate the percentage of vessels resulting from angiogenesis. Notably, no significant differences were detected between control limb microvascular density values, although these values were somewhat elevated relative to similar measures obtained from another mouse strain (29). In summary, posts ischemic angiogenesis was significantly elevated in FSAVPR-treated Cx37⁻/⁻ mice (154 ± 17%) compared with FSAVPR-treated WT mice (112 ± 6%) and sham-treated Cx37⁻/⁻ controls (106 ± 15%).

A second measure of microvascular density was also used, wherein capillary length density was estimated by stereological analysis (method 2); this method has the advantage of quantifying vascular area fraction, rather than merely microvessel number, and thus may be more sensitive to changes in network structure. In addition, this technique minimizes artifacts associated with differential tissue spreading during the fixation and sectioning process that typically vary from sample to sample. Consistent with our findings shown in Fig. 2B, surgical limb microvascular length density, as assessed by calculating mean estimated microvascular length density by method 2, was also found to be significantly increased in FSAVPR-treated Cx37⁻/⁻ animals (1,855 ± 114 mm/mm³) compared with FSAVPR-treated WT animals (1,434 ± 120 mm/mm³) and sham-treated Cx37⁻/⁻ controls (1,493 ± 83 mm/mm³; Fig. 2C). No significant differences were detected between the estimated length densities obtained for control limbs of WT animals (FSAVPR: 1,235 ± 91 mm/mm³ and sham: 1,465 ± 74 mm/mm³) and Cx37⁻/⁻ animals (FSAVPR: 1,295 ± 120 mm/mm³ and sham: 1,369 ± 113 mm/mm³). Similar to our calculations using method 1, angiogenesis was significantly increased in FSAVPR-treated Cx37⁻/⁻ mice (151 ± 12%) relative to FSAVPR-treated WT mice (118 ± 14%) and sham-treated Cx37⁻/⁻ controls (111 ± 14%). Both method 1 and method 2 revealed that posts ischemic angiogenesis (in %) was increased by ~50% in Cx37⁻/⁻ animals relative to WT animals. However, the validity of this increase depends on there being no differences in skeletal muscle fiber size between genotypes that might otherwise arise from genotype-related differences in atrophy or hypertrophy of the ischemic or control limbs.

To eliminate genotype-specific differences in muscle atrophy as a potential artifact in our determinations of microvascular density, we assessed skeletal muscle fiber cross-sectional area in surgical and control limb tissue of FSAVPR- and sham-treated WT and Cx37⁻/⁻ mice. As expected, FSAVPR-treated animals of either genotype experienced a decrease in surgical limb muscle fiber cross-sectional area (vs. that of the control limb; Fig. 2D), suggesting that skeletal muscle atrophy and remodeling were induced by FSAVPR; no such reduction in surgical limb muscle fiber cross-sectional area was observed in sham-treated animals. Importantly, no genotype-specific differences in muscle fiber cross-sectional area were observed in either sham- or ischemia-induced animals, suggesting that the increased post-ischemic microvascular density of Cx37⁻/⁻ animals shown in Fig. 2, B and C, is due to enhanced microvascular growth, not reduced skeletal muscle remodeling.

Thus, our data support the conclusion that Cx37 expression in the WT mouse limits microvascular angiogenesis under the growth stimulatory conditions created by severe hindlimb ischemia.

Although angiogenesis is typically induced in models of hindlimb ischemia (31) and is required for postischemic limb survival and recovery (22), increases in microvascular density are not typically observed until several days postsurgery and become maximal 7–10 days after the onset of ischemia (29, 31). Thus, enhanced angiogenesis in the Cx37⁻/⁻ mouse is unlikely to fully explain the improved posts ischemic collateral recovery observed in this animal after FSAVPR (Fig. 1), particularly at early postsurgical time points. Collateral vessels, which undergo immediate vasodilation and subsequent outward remodeling after the onset of ischemia, also contribute to postischemic limb survival and recovery. The data shown in Fig. 2 suggest that Cx37 limits angiogenesis and that deletion of this connexin enhances microvascular density; thus, the absence of Cx37 might also be predicted to enhance other vascular growth mechanisms, including vasculogenesis (resulting in increased native collateral number) and arteriogenesis (resulting in increased post ischemic collateral remodeling). Elevated collateral number would be expected to facilitate the immediate postsurgical return of blood flow to ischemic tissue in Cx37⁻/⁻ animals compared with WT mice immediately, since a greater number of vessels are available to acutely vasodilate and reperfuse distal tissue. In contrast, enhanced arteriogenesis would be expected to improve long-term post ischemic recovery by increasing the lumen size of remodeled collateral vessels to a greater extent than in WT animals, thereby supporting greater peripheral flow at later time points.

In previous studies of the hindlimb circulation, multiple collateral vessels were observed to connect the gracilis feed artery to the downstream branches of the femoral and saphenous arteries (see Fig. 3A). In the FSAVPR model, the femoral artery and vein are ligated proximal to the gracilis artery branch, thereby eliminating gracilis collateral vessels from facilitating in hindlimb recovery. Although other collateral vessels are likely remodeled to support recovery of surgical limb flow in the FSAVPR model, their locations (origins and insertions) have not been well documented. Thus, to examine collateral function in Cx37⁻/⁻ animals, we implemented the FAL model of hind limb ischemia, which preferentially shunts blood flow through native collaterals in the gracilis muscle and stimulates remodeling of these vessels over time (Fig. 3A). To confirm the induction of ischemia by FAL, we used laser-Doppler flow imaging to compare surgical and control limb perfusion before and 30 min after FAL (Fig. 3B). Mean surgical limb perfusion and total limb flow (limb perfusion normalized to perfused area) were both observed to be significantly reduced at 30 min post-FAL in WT and Cx37⁻/⁻ mice relative to presurgical values (Fig. 3, C and D). Additionally, posts ischemic perfusion and blood flow were both significantly improved in Cx37⁻/⁻ mice by ~20% at 30 min post-FAL compared with WT animals, consistent with an increase in native collateral numbers in animals lacking Cx37 expression. After the induction of hindlimb ischemia via FAL, Cx37⁻/⁻ and WT animals were allowed to recover for 21 days to maximize post ischemic collateral density due to remodeling. Throughout this period, recovery of surgical limb perfusion as well as the qualitative assessment of hindlimb appearance and...
use (Table 2) were monitored at multiple time points. Although FAL induced a modest decrease in whole animal weight on day 1 (Fig. 4A), animal weight recovery over the subsequent 21 days in both WT and Cx37−/− animals to measures equal to (or greater than) presurgical values, suggesting that postsurgical animals maintained adequate overall health. No differences in animal weights were observed between FAL-treated WT and Cx37−/− animals at any postsurgical time points, suggesting that overall animal weight was not significantly or differentially affected by the FAL model. As shown in Fig. 4B, FAL reduced surgical limb perfusion on day 1 postsurgery, but total limb flow to the surgical limb recovered to comparable levels (~80% of presurgical levels) in both Cx37−/− and WT animals, indicating a robust vascular remodeling response induced in this model. Consistent with our results from the FSAVPR model (5), total limb flow was significantly less reduced in Cx37−/− animals (vs. WT mice) on days 1 and 3 after the induction of ischemia by FAL. Despite these early differences in total limb flow, no significant impact of the FAL model could be detected on hindlimb appearance scores in either WT or Cx37−/− animals (Fig. 4C); in contrast, differences in postsurgical limb appearance were detected between WT and Cx37−/− mice in the more severe hindlimb ischemia model, FSAVPR, at all postsurgical time points (5) (Fig. 1). Notably, however, 25% (3 of 12) of FAL-treated WT animals experienced necrosis or auto-amputation of surgical limb tissue by day 14 post-FAL compared with 0% (0 of 11) of Cx37−/− animals (Fig. 4C, inset). This incidence of necrosis is similar to that of FSAVPR-treated WT animals [22% (2 of 9), reported in Ref. 5], despite the reduced severity of the FAL surgical model. These findings suggest a differential impact of FAL (and FSAVPR)-induced ischemia on tissue health in Cx37−/− mice relative to WT mice. Consistent with this idea [and similar to our observations in the FSAVPR model (Fig. 1B)], Cx37−/− animals experienced significantly reduced impact on, and more rapid recovery of, surgical limb use relative to WT controls (Fig. 4D) after FAL, even on day 1, when median limb use scores appeared to be identical (Fig. 4D, inset). Taken together, these data demonstrate the diminished severity of ischemia induced in Cx37−/− mice by the FAL model of hindlimb ischemia, consistent with an increased native collateral number and/or improved remodeling of these vessels (arteriogenesis) in Cx37−/− animals.

To assess the effect of Cx37 deletion on arteriogenesis, we used a radioopaque microfill to visualize vessel number, diameter, and tortuosity in the gracilis vasculature of the surgical limbs of Cx37−/− and WT animals 21 days after FAL. Unfortunately, neither X-ray angiography nor computer-assisted tomography (CT) provide sufficient anatomic information to permit the distinction of collateral vessels from noncollateral vessels. Consequently, all gracilis vessels (including, but not limited to, gracilis collaterals) were counted and characterized in FAL-treated and control limbs of our animals. By X-ray angiography (Fig. 5A) and CT (Fig. 5B), multiple gracilis arteries, including populations of vessels with larger diameters (Fig. 5C) and exhibiting the characteristic tortuosity of remodeled collaterals, were identified in the surgical limbs of both WT and Cx37−/− animals. In contrast, no tortuous vessels could be seen in X-ray angiographs of the contralateral control limbs of these animals (data not shown). Because more gracilis vessels could be visualized by X-ray angiography compared with micro-CT (Fig. 5, A and B), we elected to use X-ray images for subsequent quantification. As shown in Fig. 5D, a significant increase in the mean vessel tortuosity ratio was detected in FAL-treated limbs of Cx37−/− mice relative to vessels of the contralateral control limbs (1.13 ± 0.02 vs. 1.02 ± 0.001) and to FAL-treated limbs of WT mice (1.06 ± 0.01). This corresponded to a significant ~10% increase in...
FAL-induced gracilis vascular tortuosity (111 ± 1.3% vs. 103 ± 1.1%) in Cx37−/− animals relative to WT animals. Thus, in addition to the effect of Cx37 on microvascular growth and native collateral development, Cx37 also appears to regulate the remodeling of large collateral vessels. There was also a significant increase in the number of vessels observed in the surgical limbs of Cx37−/− animals relative to both the contralateral control limb of the same animal (16 ± 2 vs. 8 ± 1) and the surgical limbs of WT animals (9.3 ± 1.3; Fig. 5E). This corresponded to a nearly twofold increase in the gracilis vessel density of the surgical limb compared with WT animals (208 ± 14% vs. 117 ± 24%) as a result of the FAL procedure. These findings suggest an increase in both arteriogenesis and vasculogenesis in Cx37−/− animals.

As mentioned, X-ray angiography and CT do not provide sufficient anatomic information to allow collateral vessels to be distinguished from other vessels in the hindlimb circulation. Furthermore, because collateral vessels are typically small, unperfused arteriolar-arteriolar connections, unremodeled collateral vessels of the hindlimb are frequently difficult to resolve using X-ray-based techniques. Thus, to address whether the native collateral number was enhanced in Cx37−/− animals relative to WT animals, we elected to assess the characteristics of native collaterals in the pial circulation, a vascular bed that is readily visualized grossly and that has previously been used by other groups to characterize the extent of the native collateral vasculature. Findings in this vascular bed have been shown to correlate with collateral characteristics in peripheral skeletal muscle (3). The pial circulation of WT and Cx37−/− mice was marked using a coloured microfill, and the number of collateral vessels connecting the MCA, PCA, and ACA branches was quantified (Fig. 6). As shown in Fig. 6B, Cx37−/− animals experienced a significant increase (39 ± 2 vs. 29 ± 2) in the total number of pial collaterals compared with WT animals. These results suggest Cx37 deletion enhances collateral formation in the pial circulation and further lend support to the conclusion that the native collateral number is increased in the hindlimbs of these animals. Because there currently is little evidence supporting postnatal growth of collaterals de novo (for a review, see Ref. 11), these findings imply that Cx37 exerts a growth-suppressive role on collateral formation in the developing embryo. Thus our data suggest that in the Cx37−/− embryo, absence of the growth-suppressive effect of Cx37 enhances native collateral development, which results in increased numbers of collaterals in the adult animal. These collateral vessels would be expected to dilate immediately, and subsequently remodel, in response to upstream arteriolar ligation to support enhanced recovery of the ischemic limb in the Cx37−/− versus WT animal.

Fig. 4. Surgical limb recovery is enhanced in Cx37−/− animals after the induction of ischemia by FAL. A: after the induction of unilateral hindlimb ischemia by FAL surgery (see Table 1 for sample sizes, male/female ratios, and mean initial animal weights), animal weight decreased moderately at 24 h postsurgery but recovered over the subsequent 3 wk, indicating that there were no significant (genotype specific) adverse effects of surgical manipulation on the overall health of our animals. No significant differences were detected between WT and Cx37−/− animals at any time point assessed. B: total surgical limb flow (vs. the contralateral control limb) remained depressed in both WT (n = 19) and Cx37−/− (n = 16) animals at 24 h post-FAL but showed improvement by postsurgical day 21, indicating recovery of flow by vascular remodeling. Post-FAL limb flow on days 1 and 3 was significantly better in Cx37−/− animals relative to WT controls. A subset of these animals, the cohort described in Table 1 (WT: n = 12, Cx37−/−: n = 11), was assessed for recovery of surgical limb appearance and use. C: no significant differences in limb appearance scores were detected at any post-FAL time point, although 25% (3 of 12) of WT animals experienced necrosis of the surgical limb compared with 0% (0 of 11) of Cx37−/− mice (inset). D: significant (*) improvements in surgical limb use scores were detected on post-FAL days 1, 3, 5, and 7 in Cx37−/− animals relative to WT animals by a Wilcoxon rank-sum test, despite similar appearances in median score values on day 1. Inset: scores from post-FAL day 1 reveal the range of values collected at this time point. Shaded numbers indicate the number of animals receiving a particular score; data are missing for one Cx37−/− animal at this time point.

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The results of the present study represent the first comprehensive assessment of the effect of Cx37 deletion on vascular growth and remodeling in the intact animal and are the first to specifically identify a vascular growth-related phenotype in the Cx37^{-/-} mouse after ischemia. Our results are consistent with several previous studies performed in cell culture, but contradict others. Cx37 expression has been reported to be strongly upregulated in confluent cultured microvascular ECs, but was downregulated in subconfluent, proliferating ECs, suggesting proliferation and Cx37 expression are incompatible in ECs (17). We (1) previously reported that expression of mouse Cx37, but not expression of either Cx43 or Cx40, limits proliferation of an otherwise highly proliferative cancer cell line. However, expression of human Cx37 does not appear to exert a similarly potent growth-suppressive effect in HeLa cells (13), although a recent report by Morel et al. (21) has suggested that the Cx37–319P isoform is marginally growth suppressive in this cell type. Whether these contradictory observations are due to cell type-specific differences, polymorphism-related effects, or species-specific differences between the human versus mouse Cx37 sequence remain unclear. Furthermore, phenotypic changes induced by short- or long-term culture

Fig. 5. Increases in remodeled gracilis collateral number and tortuosity were observed in Cx37^{-/-} animals compared with WT controls. A: 21 days post-FAL, the hindlimb vasculature was visualized by X-ray angiograms in WT and Cx37^{-/-} animals, revealing in the surgical limb tortuous gracilis vessels characteristic of remodeled collaterals typically observed after this surgical model (scale bar = 5 mm). B: at the same time point, the hindlimb vasculature was imaged using microcomputed tomography (micro-CT; scale bar = 5 mm). Images of the same Cx37^{-/-} hindlimb sample are represented for each technique, whereas the WT images are from two different animals. C: gracilis artery diameters were quantified in X-ray angiographs to confirm the induction of collateral remodeling. In the surgical and contralateral control (i.e., unmanipulated) limbs of WT and Cx37^{-/-} animals (n = 4 animals/group, with animal identification numbers as shown), mean diameter values did not appear to be different; however, a population of large (80- to 120-μm diameter) vessels were uniquely detected in the surgical limbs of WT and Cx37^{-/-} animals that were not observed in angiographs taken from the control limbs of the same animals, confirming the induction of collateral remodeling. D: a significant (*) increase in gracilis vessel tortuosity in the surgical limbs of Cx37^{-/-} mice relative both to the contralateral control limb and ischemia-induced WT limbs was detected. E: gracilis vessel number was also significantly (*) increased in the surgical limbs of Cx37^{-/-} mice relative to both the contralateral control limbs of these animals and the surgical limbs of WT mice.
conditions that fail to mimic in vivo conditions (including altered presence or absence of flow, substrate, neighboring cells, or growth factors) complicate the interpretation of cell culture data and limit their applicability to the in vivo setting. Nonetheless, it remains uncertain whether human Cx37, like mouse Cx37, might regulate microvascular growth in vivo.

While the mechanism(s) underlying the growth-suppressive role of Cx37 in the vasculature remain unclear, evidence from several studies suggests that intact gap junction channel and/or hemichannel function may be required for Cx37-mediated growth suppression. We (9) recently demonstrated that loss of Cx37 channel function by mutation of Thr154 to an alanine [a dominant negative mutation that prevents gap junction channel (and likely hemichannel) function] eliminated the growth-suppressive effect of Cx37 in Rin cells. Consistent with the importance of channel function, Wong et al. (33) recently suggested that the atheroprotective effect of Cx37 on circulating monocyte adhesiveness might involve transmembrane passage of ATP via Cx37 hemichannels, implicating Cx37 hemichannel function in its cell regulatory effect(s), at least when this Cx is expressed in lymphocytes. Wong et al. (33) further demonstrated that the S319P polymorphism, located on the Cx37 COOH-terminus, also modulates the effect of Cx37 on monocyte adhesion. This finding suggests that Cx37 activity may also require regulation by the Cx37 COOH-terminus, which contains predicted binding sites for growth-related kinases PKC and MAPK (21). Interestingly, the S319P polymorphism has also been associated with an altered incidence of hemangiosarcoma, a highly invasive endothelium-derived cancer, in human patients (25). Taken together, these studies suggest that both an intact Cx37 channel domain, as well as regulatory domains located in the Cx37 COOH-terminus, might be required for Cx37 to limit endothelial proliferation. Additional truncation and site mutation experiments of the Cx37 protein remain necessary to resolve these questions. Furthermore, whether Cx37 serves to antagonize the initial growth stimulus mediated by vascular growth factor production or whether Cx37 expression increases subsequent to stimulation of EC proliferation to limit excessive cell growth remains unclear.

In addition to the hypothesized growth-suppressive role of Cx37 in the endothelium of small and large vessels, Cx37 may also regulate arteriogenesis by affecting vascular smooth muscle cells (SMCs). Cx37 expression has been reported in SMCs (10), where its expression is surprisingly upregulated [not downregulated, as occurs in proliferating ECs of large vessels (8) or cultured microvessels (17)] after the induction of arteriogenesis (2). Whether Cx37 expression limits SMC proliferation in a manner critical for coordinated collateral remodeling, whether Cx37 facilitates the coordination of mesenchymal cell recruitment [a process already shown to involve another vascular connexin, Cx43 (12)], or whether Cx37 participates in a currently unidentified mechanism in smooth muscle remodeling remain unclear. The data presented herein are unable to resolve EC versus SMC effects of Cx37 on large vessel remodeling; additional studies involving an endothelium-specific Cx37 knockout mouse are required to further address this question. However, that Cx37 expression appears to limit growth of the microvasculature, which at the capillary level lacks a smooth muscle layer, implies that the effect of Cx37 on the endothelium may be of greater impact on large vessel remodeling than any additional regulatory effects of Cx37 on the SMC layer.

Whereas the data presented in this study suggest that mice lacking the growth-suppressive effect of Cx37 in the vasculature renders these animals resistant to ischemic damage, it remains unclear whether antagonism of Cx37 activity would be therapeutically beneficial to patients with ischemic disease (stroke or myocardial infarction) or other forms of vascular disease. Cx37 homozygous animals might be predicted to have improved rates of exercise-induced angiogenesis associated with increased vascular proliferative capacity. However, based on the reported vascular hyperproliferation reported in this study, Cx37 homozygous mice may also experience enhanced tumour growth associated with increased proliferative capacity of the native vasculature. Furthermore, it remains uncertain whether the increased vascular proliferation observed in the Cx37 homozygous mouse produces a normal, or fully functional, vascular network. Aggressive angiogenesis during carcinogenesis associated with excessive neoplastic production of vascular growth factors results in a characteristically abnormal tumour vasculature that contains shunts and blind ends that produce localized downstream hypoxia (6, 24). A more comprehensive three-dimensional characterization of the vascular networks of WT and Cx37 homozygous mice, beyond the scope of this present study, would be required to resolve whether the increased vascular proliferative capacity of the Cx37 homozygous mouse might also be associated with the formation of inefficient and abnormal vascular networks both natively and under ischemic conditions.
In summary, we report that Cx37<sup>−/−</sup> mice develop a more extensive vasculature under three conditions that stimulate the production of vascular growth factors: developmental vasculogenesis, ischemia-induced collateral remodeling (arteriogenesis), and ischemia-induced angiogenesis. Notably, our findings demonstrating an enhanced native collateral number in the pil circulation represent the first report of an anatomic blood vascular phenotype in the Cx37<sup>−/−</sup> mouse. In addition, our data suggest that vascular Cx37 expression is required for the appropriate regulation of vascular growth via regulatory mechanism(s) active in both developing animals and after injury in the postnatal vasculature and common to both large and small vessels, despite the structural differences of their respective vascular walls.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES
