Heterocellular Contact at the Myoendothelial Junction Influences Gap Junction Organization

Brant E. Isakson, Brian R. Duling

Abstract—Heterocellular communication between vascular smooth muscle cells (VSMC) and endothelial cells (EC) at the myoendothelial junction (MEJ) is a critical part of control of the arteriole wall. We have developed an in vitro model of the MEJ composed of primary cultures of murine EC and VSMC. Immunocytochemistry and immunoblots demonstrated Cx37 and Cx43 in both cell types, whereas Cx40 was found only in EC. Cx37 was excluded from the MEJ in both EC and VSMC. Connexin composition as well as functionality of the gap junctions at the MEJ was assessed by measuring diffusional transfer of biocytin and Cy3. Using connexin-specific blockers and manipulations of expression of individual connexin proteins, we confirmed that Cx37 is not a part of EC–VSMC coupling, and we demonstrated that heterotypic gap junctions are functional at the MEJ. We speculate that specific gap junction organization may be a vital component of EC–VSMC contact at the MEJ. (Circ Res. 2005;97:44-51.)

Key Words: gap junctions ■ connexin ■ myoendothelial junctions ■ endothelial cells ■ smooth muscle cells

Heterocellular communication, between and among cells of the vessel wall, plays a critical role in many aspects of vascular function and pathology.1,2 One form of communication between endothelial cells (EC) and vascular smooth muscle cells (VSMC) appears to be via extensions of the 2 cell types that pass through the internal elastic lamina and make contacts called myoendothelial junctions (MEJ).3–5 Gap junctions at the points of cell–cell contact in the MEJ form pathways for flow of second messengers or electrical current between the two cells,4,6–8 and this communication is critical in unifying their function.

Although the physiological importance of the MEJ is well known, research on this structure is limited because of the anatomical location of MEJ, limited access to both cell types in vivo, bioavailability of inhibitors in vivo, and isolation of sufficient quantities of protein from both cell types.2 In view of these complexities, an in vitro model would be highly desirable. We, therefore, developed such a model from primary cultures of mouse EC and VSMC. We used immunoblots to identify the connexins expressed and immunocytochemistry to identify connexin proteins trafficked to the MEJ. In addition, we measured movement of diffusible solutes through the gap junctions at the MEJ and used a combination of gap junctional blockers including connexin mimetic peptides. These data allowed us to ascertain the functional and spatial organization of connexins at the MEJ.

Materials and Methods
An expanded Materials and Methods section is available in the online supplement at http://circres.ahajournals.org.

Animals
All procedures and protocols in this study were approved by the University of Virginia Animal Care and Use Committee. All mice (including Cx37+/+ and Cx40−−; kind gifts of D.L. Paul8,9) were on a C57Bl/6 background.

Cell Culture
Isolation of mouse EC was based on methods described by Srinivasan et al10 and Shi et al,11 with modifications. EC were used between passages 2 and 4. Isolation of VSMC was based on the method of Owens et al,12 with modifications. VSMC were used between passages 2 and 3.

A vascular cell coculture was created by inverting a FBS precoated polyester Transwell insert (Corning) and plating VSMC at 1×106 cells/insert. After 4 days, the insert was turned over and the upper membrane was plated with EC at 1×106 cells/insert. The coculture was allowed to stabilize for at least 72 hours.

Immunoblotting
Cell lysates were isolated with laemmli buffer, run on an SDS-PAGE gel, and transferred to polyvinylidene difluoride. Blots were developed using chemiluminescence (Pierce) and reprobed as needed (Chemicon).

Immunocytochemistry
Vascular cell cocultures were incubated for 30 minutes in supplemented PBS, primary antibodies (Abs) in supplemented PBS, washed with supplemented PBS, and incubated with corresponding secondary Abs in PBS. Samples were washed with PBS and imaged with a ×60 water immersion objective (0.90 NA) on an Olympus BX50WI confocal microscope under the control of Fluoview software.

Membrane Markers
To mark the cellular extensions into the Transwell, the membrane dyes FM 1-43FX and FM 4-64 (Molecular Probes) were applied to...
the VSMC or EC, respectively (30 μmol/L in Hanks' balanced salt solution [HBSS] with 25 mmol/L HEPES, 1% dimethyl sulfoxide).

**Dye Transfer**

EC were coloaded with biocytin (5 mg/mL; 357 Da, neutral; Molecular Probes) or Cy3 (1 mg/mL; 767 Da, Z̄=1; Amersham Biosciences) and rhodamine-dextran (3 mg/mL; 4000 Da; Molecular Probes) or albumin coupled with fluorescein isothiocyanate (FITC) (3 mg/mL; 69,000 Da; Sigma) using a pinocytotic uptake method (Molecular Probes). Strepavidin 488 (1:1 binding biocytin; Molecular Probes) was used to detect biocytin in the EC and VSMC. Using Fluoview software, pores were identified with a 100 water immersion objective and a measurement rectangle (0.8 μm × 11 μm) was placed over a randomly selected pore that covered the entire focal plane of the pore. A Z-stack consisting of 4 successive XY sections in 0.2 μm steps was performed and then a computed image rotated 90 degrees (online Figure I). Using the reconstructed image, and starting at the basal side of the EC (x=0), the mean pixel intensity from XZ sections was obtained at 1-μm intervals. The pixel intensity at each point along the pore length was normalized to the maximum pixel intensity in the control conditions.

**Gap Junction Inhibitors**

Connexin-mimetic peptides are synthetic peptides corresponding to sequences on the extracellular loops of connexin proteins (for review, see Evans and Boitano14). Gap2737,43 or gap2740 (ADI and EP scientists).}

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**Figure 1.** Vascular cell coculture sustains homogenous monolayers of EC and VSMC on either side of a Transwell insert. VSMC (A through D) were plated on the under side of the Transwell inserts (A). Immunoblots from VSMC were removed from the vascular cell coculture or from culture dishes of VSMC stained for desmin (B, lanes 1 and 2). EC from vascular cell coculture or pure cultures are desmin negative (B, lanes 3 and 4). En face views of the VSMC in vascular cell coculture with EC showed no staining for VE-cadherin (C). α-Smooth muscle actin was prevalent throughout the VSMC monolayer (D). EC (E through H) were plated on the top side of the Transwell insert (E). The same blot from B above was stripped and reprobed for PECAM-1 (F). The VSMC from the inserts or the culture dishes were negative for PECAM-1 (F, lanes 1 and 2), whereas EC from both vascular cell coculture or culture dishes were positive (F, lanes 3 and 4). En face views of EC on the inserts demonstrated extensive staining for VE-cadherin (G) but not α-smooth muscle actin (H). Bar in C is 20 μm and representative for C, D, G, and H.

**Figure 2.** EC and VSMC make cell–cell contact in the pores of the Transwell insert. Transverse differential interference contrast image of a Transwell insert with EC on the top and VSMC on the bottom (A). A pore linking the EC and VSMC is highlighted by the red box and enlarged in B. Image B demonstrates EC and VSMC extensions within the pores of the insert in close physical contact. The intact Transwell was examined using an Olympus Fluoview confocal microscope. En face confocal Z-sections were made beginning at the basal side of the EC monolayer and ending at the basal side of the VSMC monolayer (5 red horizontal lines in A). Confocal sections shown in C where images were collected at 0 (basal side of EC), 2.5 μm, 5 μm, 7.5 μm, and 10 μm (basal side of VSMC) into the Transwell insert. Red in C is the lipophilic membrane marker FM 4-64 for EC; green is the lipophilic membrane marker FM 1-43FX for VSMC. Bars in A and C are 10 μm; bar in B is 1 μm.
Sigma Genosys were used at a concentration of 190 μmol/L in HBSS with 25 mmol/L HEPES. 18-Glycyrrhetinic acid (18-GA) (50 μmol/L; Sigma) was dissolved in 0.5% dimethyl sulfoxide in HBSS.

Cx43 short interference RNA (siRNA) duplexes (Qiagen; target sequence 1, 5-TAG AAG ATT CAA AGA GCT TAA-3; target sequence 2, 5-TCC CGT GGA GGT GGT ACT CAA-3; 25 nmol/L), as well as negative control siRNA (target sequence, 5-AAT TCT CCG AAC GTG TCA CGT-3; 25 nmol/L; Qiagen) were mixed with siLentFect (Bio-Rad) and transfected into EC or VSMC on the Transwell. None of the Cx43 target sequences affected total Cx37 or Cx40 protein expression (online Figure II).

Statistics
Significance was at P<0.05 and determined by one-way ANOVA (Bonferroni post hoc test); error bars are ±SE.

Results

Vascular Cell Coculture Model
Immunoblots demonstrated selective expression of desmin and α-smooth muscle actin in VSMC but not in EC (Figure 1A, 1B [lanes 1 and 2], and 1D). EC were positive for platelet-endothelial cell adhesion molecule (PECAM)-1 (Figure 2E and 2F [lanes 3 and 4]), whereas the VSMC were not (Figure 1F, lanes 1 and 2). EC also expressed VE-cadherin (Figure 1G), but not α-smooth muscle actin (Figure 1H).

In transverse sections (Figure 2A), EC and VSMC extended processes into the Transwell pores to make cell–cell contact (red box, Figure 2A and 2B). For clarification of cellular membranes at the contact points, the cell membranes of EC and VSMC were labeled with different lipophillic dyes (EC, FM 4-64, red; VSMC, FM 1-43FX, green). Red membrane was detectable 2.5 μm into the pore, and green fluorescence associated with the VSMC penetrated 7.5 μm into the pore (Figure 2C). Overlap was detectable over a distance of ≈5 μm (yellow fluorescence).

Connexin Expression
Connexin 37 was prominent in both EC and VSMC monolayers (Figure 3A and 3D) but never observed in the pores of the Transwell (Figure 3C). Cx40 was detectable only in EC (Figure 3E and 3F). Cx43 was found only in EC (Figure 3I through 3L). Immunoblots of EC (Figure 3M, lane 1) or VSMC (Figure 3M, lane 2) from the vascular coculture were probed with Cx37, Cx40, or Cx43 (M). Cx37 and Cx43 (Sigma) are both expressed in EC and VSMC, whereas Cx40 is found only in EC. β-Actin was a loading control for each blot with representative expression demonstrated. Arrowhead is 35.5 KDa for each box.
Cx40, and Cx37 were all detected in the cellular extensions (Figure 4B1 through 4B3). When VSMC were grown on both sides of the Transwell insert (Figure 4B), Cx43 and Cx37 were detected in the cellular extensions (Figure 4C1 through 4C2), and Cx40 was absent (Figure 4C3). Only in the case of EC–VSM contact was Cx37 absent from points of heterocellular contact.

Biocytin Dye Transfer
We assessed the functionality of the connexins by measuring diffusion of biocytin from EC into VSMC. En face views of the EC or VSMC on the vascular cell coculture demonstrated that biocytin could not diffuse to VSMC when 18α-GA was present but could in control conditions (Figure 5A). In an attempt to quantify cell–cell coupling at the MEJ, we examined biocytin distribution along the Transwell pore length. Biocytin was uniformly distributed along the length of the pore from EC to VSMC (Figure 5B). Biocytin movement was inhibited by 18α-GA (Figure 5B). Connexin-mimetic peptides (Figure 5B; gap2740 and gap2737,43) each produced partial blockades, and their combination was additive (Figure 5B, combo), indicating that there was participation of more than one connexin isoform in the dye transfer.

To confirm our observations that Cx37 was present in EC, but not at the MEJ, Cx37−/− EC cultured from a Cx37−/− mouse were used. There was no effect on biocytin dye transfer to wild-type VSMC (Figure 5C), and 18α-GA was effective, as were gap2737,43 and gap2740 (Figure 5C). Similar dye transfer results were obtained from wild-type EC cocultured with Cx37−/− VSMC (online Figure IIIA). In addition, Cx37 did not redistribute to the MEJ when Cx37−/− EC or VSMC were used (online Figure IV).

When the vascular cell coculture was prepared with Cx40−/− EC and wild-type VSMC, dye transfer persisted, and the effect of 18α-GA was unaltered (Figure 5D), but gap2740 was ineffective (Figure 5D). Gap2737,43 and the combination of both peptides had identical effects on biocytin transfer. Biocytin movement from wild-type EC to Cx40−/− VSMC was similar to Figure 5A (online Figure IIIB), demonstrating no role for Cx40 in VSMC.

Effect of Cx43 siRNA on Biocytin Dye Transfer
Cx43 siRNA oligonucleotides transfected into EC or VSMC were able to significantly reduce Cx43 protein expression in both cell types. Immunoblots of EC and EC transfected with nonsilencing siRNA (Figure 6A, lanes 1 and 2) demonstrated Cx43 staining. When either Cx43 siRNA target sequence 1 (Figure 6A, lane 3) or Cx43 siRNA target sequence 2 (Figure 6A, lane 4) were transfected into EC, there was significant reduction in Cx43 protein. Untreated VSMC or those transfected with nonsilencing siRNA showed Cx43 staining (Figure 6A, lanes 5 and 6). Both of the Cx43 siRNA oligonucleotides reduced Cx43 protein in VSMC (Figure 6A, lanes 7 and 8).

Transfection of Cx43 siRNA into VSMC (Figure 6B) or EC (Figure 6C) resulted in significant Cx43 protein reduction (Figure 6B and 6C) in comparison with control sections (Figure 3J and 3K) and compared well with sections in which the Abs were preabsorbed with Cx43-GST peptide (Figure 6D15). Cx37 did not redistribute to the MEJ when Cx43 siRNA was present in EC or VSMC (online Figure IV).

We loaded biocytin into wild-type EC and examined transfer to VSMC transfected with Cx43 siRNA (Figure 7A). In control conditions, the diffusion of biocytin was halted 4 to 5 μm into the Transwell. Cocultures of wild-type EC and HeLa cells demonstrated biocytin dye transfer mimicked that seen in Figure 7A (online Figure IIIIC).

Biocytin transfer persisted between EC with depleted Cx43 and wild-type VSMC (Figure 7B). Cx40−/− EC transfected with Cx43 siRNA showed reduced biocytin transfer to wild-type VSMC, similar to that seen in Figure 7A and online...
Myoendothelial junctions are thought to tightly regulate EC and VSMC communication; dye transfer, bidirectional signaling, and electrical coupling between the two cell types are well established. However, connexins involved and the signaling pathways activated are poorly understood and, at this time, there is no information as to the connexins that heterocellular contact played a key role in guiding the organization of connexins, not just on connexin expression. Moreover, dye coupling measurements, and the use of selective blockers, combined with the alteration of protein expression, all confirmed the lack of Cx37 at the MEJ. In a similar example of Cx37 in heterocellular contact, Veitch et al have demonstrated that Cx37 protein was not present in granulosa cells until oocytes were present in the culture, implying oocytes were able to induce Cx37 upregulation in another cell type. In our system, Cx37 was always present in both cell types, either apart or in heterocellular contact, implying a possible role for heterocellular contact in control of connexin protein trafficking. It is clear that more work on Cx37 protein trafficking, as demonstrated in either system, is required.

The literature provides a variety of mechanisms for selective organization of connexin hexamers. Oligomerization of connexins could hypothetically occur at different locations in the ER/Golgi secretory pathway. It is possible that when heteromeric hexamers (such as the Cx40/Cx43 hexamer described here) are formed, they alter the location of oligomerization in the secretory pathway, which may lead directly to their transport to different parts of the cell. Thus, connexin hexamers could use different transport path-
ways for targeting to different regions of the plasma membrane, such as the MEJ.

We do not believe that connexins other than Cx40 and Cx43 are involved in EC and VSMC communication in this model. However, the mRNA for Cx45 has been detected in mouse EC, and on Cx45 gene ablation from mouse, vessel formation is inhibited. In VSMC in control conditions and containing nonsilencing siRNA (A, lanes 5 and 6) demonstrated expression of Cx43 protein, whereas the addition of duplex 1 or duplex 2 to the VSMC induced Cx43 protein reduction (A, lanes 7 and 8). The blot was stripped and reprobed with β-actin to compare cell loading. The extent of the box height is 40 KDa on the bottom to 50 KDa on the top. Immunocytochemistry of Cx43 protein (red) within the pores linking EC (green, VE-cadherin; B1, C1, and D1) and VSMC (green, desmin; B2, C2, and D2) demonstrated that whether Cx43 siRNA was loaded into VSM (B) or EC (C), there was a qualitative decrease in Cx43 staining (Chemicon) in the loaded cell type. As a comparison for Cx43 reduction, Cx43 staining in wild-type EC and VSMC were blocked with Cx43 GST (D; positive staining, Figure 5J and 5K). Cx43 siRNA duplex 1 was used in B and C, although duplex 2 gave similar results (data not shown). Bar in B is 2 μm.

Figure 6. siRNA directed against Cx43 mRNA significantly inhibited Cx43 protein production in EC and VSMC. Using immunoblots, EC in control conditions and EC loaded with nonsilencing siRNA both demonstrate Cx43 expression (Sigma) (A, lanes 1 to 2), whereas the addition of Cx43 siRNA duplex 1 or duplex 2 reduced the amount of Cx43 protein (A, lanes 3 and 4). In VSMC in control conditions and containing nonsilencing siRNA (A, lanes 5 and 6) demonstrated expression of Cx43 protein, whereas the addition of duplex 1 or duplex 2 to the VSMC induced Cx43 protein reduction (A, lanes 7 and 8). The blot was stripped and reprobed with β-actin to compare cell loading. The extent of the box height is 40 KDa on the bottom to 50 KDa on the top. Immunocytochemistry of Cx43 protein (red) within the pores linking EC (green, VE-cadherin; B1, C1, and D1) and VSMC (green, desmin; B2, C2, and D2) demonstrated that whether Cx43 siRNA was loaded into VSM (B) or EC (C), there was a qualitative decrease in Cx43 staining (Chemicon) in the loaded cell type. As a comparison for Cx43 reduction, Cx43 staining in wild-type EC and VSMC were blocked with Cx43 GST (D; positive staining, Figure 5J and 5K). Cx43 siRNA duplex 1 was used in B and C, although duplex 2 gave similar results (data not shown). Bar in B is 2 μm.

Figure 7. Biocytin dye transfer from EC to VSMC was inhibited when Cx43 was reduced in the VSM and when Cx40 and Cx43 were eliminated from the EC. Biocytin was loaded into EC, and transfer through the pores to VSMC at 1-μm intervals was determined by streptavidin pixel intensity. Three different experimental paradigms were tested: biocytin transfer from wild-type EC to VSMC loaded with Cx43 siRNA (A), EC loaded with Cx43 siRNA to wild-type VSMC (B), or Cx40−/− EC loaded with Cx43 siRNA to wild-type VSMC (C). Purple lines represent biocytin transfer in control conditions (control); gray lines represent transfer in the presence of 18α-GA; red lines, transfer in the presence of gap27; blue lines, transfer in the presence of both gap27 and gap27 (combo). n indicates the number of pores examined per paradigm; symbols, *P<0.05 when compared with control at each point along the pore length; +18α-GA, #combo, ^gap27, ⬤gap27, +gap27, −gap27. Cx43 siRNA duplex 1 was used in A through C, although duplex 2 gave similar results (data not shown).
prevented these data as showing that multiple connexins existed at the MEJ, including Cx40 and Cx43 in the EC and Cx43 in the VSMC. To confirm this, we examined biocytin transfer from Cx40$^{-/-}$ EC to wild-type VSMC. Deletion of Cx40 did not result in any changes in dye transfer to VSMC. However, when gap27$^{40}$ was added, there was no inhibitory effect of the peptide. This suggested that Cx40 in EC may be a normal constituent of the gap junctions at the MEJ but that its function can be subsumed by Cx43 in its absence. The deletion of Cx43 from EC resulted in a similar pattern of dye transfer to that seen when wild-type EC and VSMC were used, indicating again that at least one connexin (likely Cx40) in the EC was still able to form gap junctions at the MEJ. In an attempt to determine exactly which connexins were present on the EC side of the MEJ, we added Cx43 siRNA to Cx40$^{-/-}$ EC. In these circumstances, all biocytin transfer from EC to VSMC was blocked. The only other instance of a complete block observed was when Cx43 protein was ablated from the VSMC (Figure 7A). These data are consistent with the overall concept that Cx40 and Cx43 from wild-type EC form functional gap junctions solely with Cx43 from wild-type VSMC.

Our data indicate that combinations of connexins form gap junctions at the MEJ. The possible connexin organizations included the following: (1) homotypic Cx43 gap junctions, (2) Cx40 hexamers from EC and Cx43 hexamers from VSMC forming heterotypic gap junctions, or (3) heteromeric hexamers of Cx40 and Cx43 from EC and hexamers of Cx43 from VSMC forming heterotypic gap junctions. We used cells from knockout mice to explore these possibilities. Elimination of Cx40 from the EC leaving only Cx43 on the EC side of the MEJ should have forced the formation of homotypic gap junctions between the two cells. We also deleted Cx43 from the EC with Cx43 siRNA, leaving only Cx40 from the EC in the MEJ, thereby forcing the formation of a heterotypic gap junction with the Cx43 from the VSMC. In each of these manipulations, biocytin dye transfer to VSMC indicated that functional gap junctional coupling remained at the MEJ (Figures 5C and 7B).

The biophysical properties of biocytin would not likely distinguish between gap junctions composed of different connexins, and we, therefore, selected Cy3, which is similar to Alexa 594 (820 Da, Z = -1) and has been demonstrated to diffuse through Cx43 homotypic gap junctions but not heterotypic Cx40–Cx43 gap junctions. We assessed Cy3 diffusion from EC that were wild-type, Cx40$^{+/+}$, or transfected with Cx43 siRNA (Figure 8) to wild-type VSMC. Only in Cx40$^{-/-}$ EC was Cy3 dye transfer to wild-type VSMC possible, ie, only in a situation in which it appeared that homotypic Cx43 gap junctions would have been present at the MEJ. The failure of Cy3 to diffuse from wild-type EC or Cx43 siRNA EC to VSMC argues that the functional gap junctions were, in this case, heterotypic. In view of the participation of Cx43 in functional coupling with VSMC when Cx40 is ablated in EC, heteromeric hexamers of Cx40 and Cx43 in EC seem to be the most likely combination that is formed at the junction.
In summary, our data suggest that heterocellular communication between VSMC and EC in this model of the MEJ is based on the spatial organization of the connexin distribution within the cells. The result is the formation of heterotypic gap junctions with a resultant charge or size selectively conferred on the transfer of solute between the two cell types (EC and VSMC). These data provide a strong rationale for future experiments in intact vessels. The expression of connexins forming heterotypic gap junctions has been observed in vivo, and studies on transformed cell lines and reconstituted hexamers have demonstrated that heterotypic gap junctions manifest differences in second messenger and dye transfer permeabilities. Therefore, we propose that spatial organization of connexins, and thus selective gap junction formation, may be used by EC and VSMC to tightly coordinate solute transfer between vascular cells and thus coordinate vascular function.

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References

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Expanded Material and Methods:

Animals

All procedures and protocols in this study were approved by the University of Virginia Animal Care and Use Committee. Cx40⁻/⁻ and Cx37⁻/⁻ mice were kind gifts of D. L. Paul, Harvard University. All mice were on C57Bl/6 background. Genotyping was confirmed by PCR of genomic DNA (e.g., ¹).

Cell Culture

Unless noted, all cell culture materials were purchased from Gibco BRL. Isolation of mouse endothelial cells (EC) was based on methods described in ²,³ with modification. Briefly, the aorta was removed from the mouse, placed in HBSS with 25 mmol/L HEPES (~ 4° C), cleaned of adipose tissue and adventitia, cut laterally into 1 mm² explants, and placed on a Matrigel matrix (BD Biosciences). The EC growth medium contained low glucose DME with D-valine substituted for L-valine, 15% dialyzed-FBS, 1% antibiotic/antimycotic, 90 µg/mL heparin, and 40 µg/mL endothelial cell growth supplement (ECGS; BD Bioscience). After three days, EC began to migrate off of the explants, which were then moved to a second dish for the cultivation of vascular smooth muscle cells (VSMC; see below). After an additional seven days on Matrigel, the explant-derived EC were removed with dispase, plated onto 60 mm dishes coated with 0.01% gelatin in PBS, and grown in the EC growth medium described above (dialyzed FBS replaced with FBS and 15 µg/mL ECGS) until confluent. EC were only used between passages 2 and 4.

Isolation of VSMC was based on ⁴ with modifications. The explants from which EC had migrated were transferred to HBSS with 1% antibiotic/antimycotic, elastase (7.0
mg/mL; Sigma) and type VIII collagenase (1 mg/mL; Sigma) and incubated for 40 min at 37° C. Next, the solution was triturated for 1 min in growth medium (DME with 15% FBS, 1% antibiotic/antimycotic), and centrifuged, and seeded onto a 60 mm FBS-coated tissue culture dish. VSMC were used between passages 2 and 3.

A vascular cell co-culture was created by inverting a FBS pre-coated Transwell insert (0.4 µm diameter pore, 10 µm thick; Corning), in a 100 mm dish (with 25 mL sterile H2O). Confluent VSMC were removed from culture dishes with 0.25% trypsin-EDTA, plated on the insert at 1x10^6 cells/insert, and grown at 37° C in an incubator with a 5% CO2 atmosphere. After 4 days the VSMC were confluent and the insert was turned over and the upper membrane surface was coated with warmed 0.1% gelatin in DME for one h. EC were removed from culture dishes with 0.25% trypsin-EDTA and plated on the upper membrane surface at 1x10^7 cells/insert. The co-culture was allowed to stabilize for at least 72 h during which time junctions between the two cell types formed MEJs.

**Antibodies**

Antibodies (Abs) used to determine cellular phenotype included: mouse monoclonal anti-alpha smooth muscle actin (Sigma A 2547), mouse monoclonal anti-desmin (Sigma D 1033), goat anti-PECAM-1 (Santa Cruz sc-1506), and goat anti-VE-cadherin (Santa Cruz sc-6458). Abs used to determine the expression of specific connexin isoforms included: rabbit anti-Cx43 (Chemicon AB1727; amino acid sequence 252-270), mouse monoclonal anti-Cx43 (Sigma C 8093; amino acid sequence 130-143), rabbit anti-Cx40 (ADI CX40-A)1, rabbit anti-Cx37 (ADI CX37B12-A)5, and rabbit anti-Cx45 (ADI CX45-A). Corresponding secondary Abs included donkey anti-goat Alexa 488, donkey anti-goat Alexa 594, goat anti-rabbit Alexa 488, goat anti-rabbit Alexa 594,
rabbit anti-mouse Alexa 488, and rabbit anti-mouse Alexa 594 (Molecular Probes). To rule out cross-reactivity commonly observed with connexin Abs, they were tested in both an in vitro system (HeLa cells transfected with Cx40 or Cx43; kind gift of E.C. Beyer, University of Chicago) and in vivo systems (Cx40<sup>-/-</sup>, and Cx37<sup>-/-</sup> mice). The specificity of the Cx43 Abs were additionally tested with Cx43 GST-peptides<sup>6</sup> (kind gift of A.M. Simon, University of Arizona and A.F. Lau, University of Hawaii).

**Immunoblotting**

Cell lysates were isolated using two methods; 1) applying liquid nitrogen to the vascular cell co-cultures, bringing the vascular cell co-cultures to room temperature, and then adding laemmli buffer (to detect connexins) or 2) applying boiling laemmli buffer to vascular cell co-cultures (to detect PECAM-1, desmin). Isolated cell lysates were snap-frozen with liquid nitrogen. Proteins from the lysate samples were exposed by repeated freeze (-80° C) and thaw (95° C) cycles, and were separated on 12.5% SDS-PAGE gels. The proteins were transferred to PVDF overnight, probed with primary Abs in 5% dry milk, and exposed to secondary Abs coupled to horseradish peroxidase. Blots were developed using chemiluminescence (Pierce). For multiple probes, the blots were stripped with Re-Blot Plus Strong (Chemicon).

**Immunocytochemistry**

Vascular cell co-cultures were prepared for immunocytochemistry by fixation in buffered 10% formalin overnight at 4° C. The Transwell inserts were prepared for sectioning by passing them through sequential increases in sucrose concentrations, embedding them in OCT, and cutting them into 10 µm frozen sections. Sections were incubated for 30 min in PBS supplemented with 4% fish skin gelatin, 0.25% Triton X-
100 and 1% serum from the source animal in which the secondary Abs were made. Next, sections were incubated with primary Abs in supplemented PBS for 2 h at room temperature, washed with supplemented PBS, and incubated with corresponding secondary Abs in PBS for 1 h at room temperature. Images with more than one primary Ab were subjected to this procedure twice with non-competitive primary and secondary Abs. Samples were washed with PBS and imaged with a 60X water immersion objective (0.90 N.A.) on an Olympus BX50WI confocal microscope under the control of Fluoview software. En face images could be obtained from both the EC and the VSMC by preparing Transwell inserts for immunocytochemistry as described above. After immunocytochemistry, the inserts were removed with a scalpel, placed on a slide, and stained with SYTOX green (Molecular Probes) in PBS.

**Membrane Markers**

To mark the cell borders and extensions, the membrane dyes FM 1-43FX and FM 4-64 (Molecular Probes) were applied to the VSMC or EC respectively (30 µmol/L in HBSS with 25 mmol/L HEPES, 1% DMSO). Using a 100X water immersion objective (1.00 N.A.) confocal images of both cell types were obtained by scanning through the specimen five times in 2.5 µm steps from EC to VSMC.

**Dye Transfer**

EC were co-loaded with biocytin (5 mg/mL; 357 Da, neutral charge, Molecular Probes) and rhodamine-dextran (3 mg/mL; 4000 Da, Molecular Probes) using a pinocytotic uptake method (Molecular Probes). After 20 min, the inserts were then placed in buffered 10% formalin and 10 µm frozen transverse sections were prepared as described above. Strepavidin 488 (Molecular Probes) was used to detect biocytin in the
EC and VSMC. Cross sections of the Transwell inserts were placed on the microscope stage, and the Transwell pores were identified with a 100X water immersion objective. Using Fluoview software a measurement rectangle (0.8 µm x 11 µm) was placed over a selected pore, including the bases of the EC and VSMC. A Z-stack of multiple scans of the rectangle was prepared (4 XY sections in 0.2 µm steps). The images were stacked and rotated 90° to reconstruct the pore (Supplementary Fig. 3). Using the reconstructed image, and starting at the basal side of the EC (x=0), the mean pixel intensity was obtained at 1 µm intervals between EC and VSMC. The pixel intensity at each point along the pore length was normalized to the pixel intensity in the control conditions at the EC end of the pore. (e.g, Fig 5A). The resulting graph represents a semi-quantitative estimate of the passage of biocytin down the cellular extensions within the Transwell insert pores and consequently through the intervening gap junctions at the MEJ.

**Gap Junction Inhibitors**

Gap2737,43 or gap2740 (ADI and Sigma Genosys) were used at a concentration of 190 µmol/L in HBSS with 25 mM HEPES. Concentrations up to 300 µM were had similar effects (data not shown). Fifty µmol/L 18α-glycyrrhetinic acid (18 alpha GA; Sigma G-8503) was dissolved in 0.5% DMSO in HBSS. Peptides were applied for 60 min and 18 alpha GA was applied for 20 min at 37° C.

Cx43 protein expression was decreased by targeting Cx43 mRNA with siRNA. Cx43 siRNA duplexes (Qiagen; target sequence #1, TAG AAG ATT CAA AGA GCT TAA, 25 nmol/L; target sequence #2 TCC CGT GGA GGT GGT ACT CAA, 25 nmol/L) were mixed with siLentFect (Bio-Rad) and transfected into EC or VSMC on the Transwell insert. The peak of Cx43 protein knockdown was at 36 h post-transfection in
both cell types (data not shown) and all measurements of function were conducted at this time. Non-silencing siRNA duplexes (Qiagen) were used as controls.

**Cy3 Dye Transfer**

Cy3 (1 mg/mL; 767 Da, -1, Amersham Biosciences) and albumin coupled with FITC (3 mg/mL; > 69,000 Da, Sigma) were co-loaded into EC using the pinocytotic method described above. After loading, cells rested for 20 minutes before being fixed with 10% buffered formalin overnight at 4°C. EC and the VSMC directly beneath were viewed en face using a 60X water immersion objective (0.90 N.A.) as described above.

**Statistics**

Significance was set at P<0.05 and determined by one-way ANOVA (Bonferroni post-test) comparing experimental conditions to control conditions at each distance along the pore length within each cell co-culture. All error bars are +/- the standard error.
Reference List


Supplementary Figure Legends:

Supplementary Figure 1: Acquisition method for determining biocytin dye transfer from EC to VSMC. DIC image of a vascular co-culture with the EC monolayer on the top, and the VSMC monolayer on the bottom, of a porous (0.4 µm) Transwell insert (A). The EC on the top of the insert were loaded with rhodamine-dextran and biocytin. The rhodamine fluorescence from dextran is demonstrated in B, whereas streptavidin coupled to Alexa-488 demonstrates the localization of biocytin in C. The red box in C is representative of a box applied to the image using Fluoview software. Sequential Z-stacks through the pore and subsequent 3D image reconstruction results in D. Image D demonstrates a sample of the xz sections measure every 1 µm down the length of the pore used to acquire pixel intensity. Bars in A are 10 µm and representative for images A-C.

Supplementary Figure 2: Cx37 and Cx40 total protein in EC or VSMC is unaltered by Cx43 siRNA. Total protein from EC (odd lanes) or VSMC (even lanes) was isolated from the vascular co-culture. Immunoblots were performed in several different paradigms: control conditions (lanes 1-2; similar to Fig 3M), when EC were transfected with Cx43 siRNA duplex #1 (lanes 3-4), or VSMC were transfected with Cx43 siRNA duplex #1 (lanes 5-6). In addition, Cx43 siRNA duplex #2 transfected into EC (lanes 7-8) or VSMC (lanes 9-10) were also examined. None of the Cx43 siRNA duplexes affected Cx37 or Cx40 protein expression. Beta-actin was used as a loading control. Each box height spans approximately 30 KDa on the bottom and 45 KDa on the top.
Supplementary Figure 3: Biocytin dye transfer from EC to Cx40<sup>-/-</sup> VSMC, Cx37<sup>-/-</sup> VSMC or HeLa cells. Biocytin loaded into EC and transfer through the Transwell insert pores to VSMC at 1 µm intervals was determined by streptavidin pixel intensity. Three different experimental paradigms were tested, including wild-type EC transfer to Cx37<sup>-/-</sup> VSMC (A), wild-type EC transfer to Cx40<sup>-/-</sup> VSMC (B), or wild-type EC transfer to HeLa cells. Purple lines represent biocytin transfer in control conditions (control); gray lines represent transfer in the presence of 18α-glycyrrhetinic acid (18 alpha GA); red lines represent transfer in the presence of gap27<sup>40</sup> (gap27<sup>40</sup>); blue lines represent transfer in the presence of gap27<sup>37,43</sup> (gap27<sup>37,43</sup>); green lines represent transfer in the presence of both gap27<sup>37,43</sup> and gap27<sup>40</sup> (combo). “n” is the number of pores examined per paradigm. Symbols represent p<0.05 when compared to control at each point along the pore length: * 18 alpha GA; # combo; + gap27<sup>37,43</sup>; ^ gap27<sup>40</sup>.

Supplementary Figure 4: Connexin 37 does not appear at MEJ, regardless of the connexin ablation. Transverse sections of vascular cell-co cultures were stained with a Cx37 antibody. Different combinations of EC and VSMC were used to identify the location of Cx37 protein. The images under the column designated “EC<sup>-/-</sup>” had a connexin deleted out of EC and were cultured with wild-type VSMC. The images under the column heading “VSM<sup>-/-</sup>” had a connexin deleted from VSMC and were cultured with wild-type EC. EC or VSMC with deleted connexins were obtained from Cx37<sup>-/-</sup> or Cx40<sup>-/-</sup> mice or cells transfected with Cx43 siRNA. Bar equals 1 µm.
Supplementary Figure 3

**Relative Dye Transfer**

**A**
EC → Cx37−/− VSM

- 18 alpha GA (n=18)
- control (n=15)
- gap27 (n=17)
- gap27,37 (n=18)
- combo (n=14)

**B**
EC → Cx40−/− VSM

- 18 alpha GA (n=14)
- control (n=14)
- gap27 (n=16)
- gap27,37,40 (n=18)
- combo (n=15)

**C**
EC → HeLa

- 18 alpha GA (n=12)
- control (n=12)