Connexin45 Regulates Endothelial-Induced Mesenchymal Cell Differentiation Toward a Mural Cell Phenotype

Jennifer S. Fang,* Cuiping Dai,* David T. Kurjiaka, Janis M. Burt, Karen K. Hirschi

Objective—The focus of this study was to investigate the role of connexin (Cx) 45 in endothelial-induced mural cell differentiation.

Methods and Results—We created mural cell precursors that stably express only Cx45 in Cx43-deficient mesenchymal cells (ReCx45), and used our in vitro model of blood vessel assembly to assess the capacity of this Cx to support endothelial-induced mural cell differentiation. Lucifer Yellow dye injection and dual whole-cell patch clamping revealed that functional gap junctions exhibiting properties of Cx45-containing channels formed among ReCx45 transfectants, and between ReCx45 and endothelial cells. Heterocellular Cx45-containing gap junction channels enabled transforming growth factor-β activation and promoted the upregulation of mural cell–specific proteins in the mesenchymal precursors.

Conclusion—These studies reveal a critical role for Cx45 in the regulation of endothelial-induced mural cell differentiation, which is consistent with the phenotype of Cx45-deficient embryos that exhibit dysregulated transforming growth factor-β and lack mural cell development. (Arterioscler Thromb Vasc Biol. 2013;33:362-368.)

Key Words: connexin • endothelial cell • gap junction • mural cell development • TGF-β

Blood vessels are composed predominantly of 2 cell types: endothelial cells (ECs) that form the luminal lining and mural cells (vascular smooth muscle cells and pericytes) that make up the surrounding medial layer. During blood vessel formation, endothelial tubes form first and govern the subsequent formation of the vessel wall via release of platelet-derived growth factor-B, which acts as a chemoattractant, and mitogen for mural cell precursors derived from the surrounding mesenchyme.1,2 On contact with ECs, newly recruited mesenchymal progenitor cells are induced toward a mural cell fate2 by EC-mediated activation of transforming growth factor-β (TGF-β).3-5 Although the process of TGF-β activation in response to heterocellular interactions is unclear,6,7 gene-targeting experiments indicate that TGF-β signaling via activation of activin-like kinase receptors8 plays a critical role in vascular development. Thus, local mesenchymal progenitors are recruited by ECs to differentiate into the mural cell layer(s) in developing vessels, likely resulting in tissue-specific and regulatory properties of mural cells.10

Observations from genetically altered mice suggest that gap junctions play a critical role in vascular development,11-13 and we showed more specifically that gap junction channel formation between ECs and recruited mesenchymal cells is required for their endothelial-induced differentiation into a mural cell phenotype.3 Gap junctions are aggregates of intercellular channels that allow the diffusion of second messengers, ions, and metabolites to the cytoplasm of adjoining cells.14 Gap junction channels that form through the docking if 2 hexameric complexes between adjacent vascular cells may be composed of one or more different types of connexin (Cx) proteins that include Cx37, Cx40, Cx43, and Cx45.5,15-17 In the adult arterial vasculature, ECs of large vessels predominantly express Cx37 and Cx40, whereas Cx43 expression is largely in the microvasculature and mural cells.15,18 Although Cx45 is modestly coexpressed with Cx43 in the medial layer of adult vessels,19 it does not appear to be a major contributor to gap junction function in postnatal vasculature.18,20,21 However, Cx45 is highly expressed in the developing vasculature in both ECs and smooth muscle cells,22,23 where it appears to be critical for mural cell investment of EC tubes; Cx45-deficient mice die midgestation, in part, because of failure of vascular smooth muscle formation.11 Interestingly, although Cx43 is often coexpressed with Cx45 during development,22,23 it cannot compensate for loss of Cx45 during early stages of blood vessel formation. Similarly, Cx45 does not compensate for lack of Cx43 at later stages of development; mice deficient for Cx43 die perinatally from severe cardiovascular malformations.12 Thus, Cx43 and Cx45 are both critical for proper vascular development, although their exact function(s), distinct or similar, at various stages of development have not been clearly delineated.

In previous studies, we demonstrated that Cx43 is highly expressed by ECs, as well as mesenchymal cells, that function
as mural cell precursors. Furthermore, Cx43-mediated coupling between ECs and mesenchymal cells promotes activation of TGF-β that enables endothelial-induced mural cell differentiation. In this study, we investigated whether Cx45, which is also expressed by ECs and mesenchymal cells, although to a lesser extent, also regulates mural cell differentiation via similar mechanisms. We generated mesenchymal cells that stably express Cx45 (ReCx45) in parental cells that lack Cx43 (derived from Cx43–/– mice), and found that they formed functional gap junctions among themselves and with ECs. Heterocellular Cx45-containing gap junctions supported TGF-β activation and promoted the upregulation of mural cell-specific proteins in the mesenchymal cells. These results reveal a critical, and previously undefined, role for Cx45 in the regulation of mural cell differentiation that likely underlie the lethal defects observed in Cx45-deficient embryos, which exhibit dysregulated TGF-β and lack mural cell development.

Materials and Methods

Extended methods are provided in the online-only Data Supplement.

Cell Culture

Cx43-expressing (Cx43wt) and Cx43-deficient (Cx43–/–) mesenchymal cells were used. Cx43–/– mesenchymal cells were also transiently transfected with full-length Cx45 (ReC45; see below). ECs and smooth muscle cells were isolated from bovine tissue. In some experiments, cells were plated on 0.25 to 1 μg/cm² fibronectin (Sigma #F4759) at 37 °C.

Generation of Cx45-Expressing Mesenchymal Cells

Cx45 cDNA in a pCl-neo vector was stably transfected into Cx43–/– mesenchymal cells (ReCx45). Individual clones of stable transfectants were selected for further study.

Assessment of Gap Junction Communication

Gap junction intercellular communication was assessed by scrape loading, dual dye injection, and whole-cell patch clamping, as detailed in the online-only Data Supplement.

Assessment of Endothelial-Induced Mural Cell Differentiation

ECs and mesenchymal cells were cultured alone or in coculture for 48 hours, and Western blot and immunofluorescent techniques were used to assess mural cell differentiation, as described.

Measurement of Activated TGF-β

An established luciferase bioassay was employed to measure levels of activated TGF-β, as described.

Treatment With Exogenous TGF-β1 or TGF-β Blocking Antibodies

Cells were exposed for 48 hours to either 1 ng/mL human TGF-β1 (R&D #100-B-001) or 10 μg/mL TGF-β-blocking antibodies that target all isoforms of TGF-β (Genzyme #1836-01), as described.

Assessment of Extracellular Matrix Deposition

Solo cultures or cocultures of PKH26-labeled ECs and unlabeled mesenchymal cells were plated for 48 hours onto glass coverslips and assessed for fibronectin (BD #610077, 1:250) or fibrillin (Santa Cruz #sc-7540, 1:100) expression by immunofluorescence. Five random fields from 4 to 12 experiments were quantified for extracellular matrix (ECM) deposition.

Results

We previously showed that Cx43-deficient mesenchymal cells fail to form functional gap junctions with ECs, and undergo mural cell differentiation on heterocellular contact. Re-expression of Cx43 in Cx43–/– mesenchymal cells restored gap junction formation, TGF-β activation, and endothelial-induced mural cell differentiation. Observations from the Cx45 knockout animal suggest that this Cx is necessary for mural cell development during embryogenesis. Using our in vitro model, we investigated whether Cx45 plays a direct role in endothelial-induced mural cell differentiation.

Stable Expression of Cx45 in Cx43–/– Mesenchymal Cells

To determine whether Cx45 can regulate endothelial-induced mural cell differentiation, we generated mesenchymal cells that expressed only Cx45 (ReCx45). The Cx43–/– mesenchymal cells represent an excellent model as its primary Cx has been removed, and the remaining low levels of Cx45 are not detectable by Western blot analysis and do not support heterocellular communication of injected dyes or mural cell differentiation. In this study, full-length Cx45 cDNA was transfected into Cx43–/– mesenchymal cells, and stable puromycin-resistant clones were selected. Two clones (ReCx45-1 and ReCx45-3) expressed ~45 kDa protein detected by anti-Cx45 antibodies (Figure 1A). ReCx45-1 expressed the highest levels of Cx45, and ReCx45-3 expressed levels that were ~90% of ReCx45-1; both of these clones were used for all

Figure 1. Stable expression of connexin (Cx) 45 in Cx43–/– mesenchymal cells. A, Western blot analyses demonstrated that Cx43–/– cells transfected with the empty vector did not express detectable Cx45 protein. Stably transfected clones, ReCx45-1 and ReCx45-3, expressed significantly higher levels of Cx45 protein, whereas the ReCx45-2 clone did not. B, Immunocytochemistry of ReCx45-1 (and ReCx45-3; not shown) cells revealed significant Cx43 expression (red), which was not observed in parental Cx43–/– mesenchymal cells (scale=50 μm; nuclei=blue).
subsequent studies. No Cx45 expression was evident in Cx43−
mesenchymal cells transfected with empty vector, or in the
ReCx45-2 clone (Figure 1A). Immunofluorescent analysis of
ReCx45-1 (and ReCx45-3; not shown) revealed abundant
Cx45 signal compared with parental Cx43−/−
cells (Figure 1B).

Heterotypic Communication Between
Cx45 Mesenchymal Cells and ECs
To determine whether Cx45 mediates intercellular
communication between mesenchymal cells and ECs, dye-
coupling and dual whole-cell voltage-clamping techniques were
used. Homocellular dye-coupling was assessed in confluent
monolayers of ReCx45 mesenchymal cells, using a scrape-
loading assay (data not shown).26 Uptake of the gap junction-
permeable Lucifer Yellow dye was significantly increased
(by ≈25%; n=3–6), compared with Cx- and communication-
deficient Cx43−/−
mesenchymal cells. To determine whether
dye-coupling was retained in ReCx45 cells when cocultured
with ECs, we performed microinjection of Lucifer Yellow.
The heterocellular nature of assessed cell pairs was ensured by
labeling one cell type with a fluorescent dye (either PKH26 or
DiI) and by selecting cell pairs that included a fluorescent and a
nonfluorescent cell for analysis. No dye- or electrical-coupling
could be detected in cocultures of ECs with parental Cx43−/−
mesenchymal cells.3 In contrast, 20 of 102 (19.6%) ReCx45
cells were dye-coupled to at least 1 neighboring EC (Figure 2A).

Because Cx45-comprised junctions are cation-selective,27
and thus, in general, poorly permeated by anionic dyes, this
level of dye-coupling might be indicative of a higher incidence
of electrical coupling. Thus, we used dual whole-cell voltage-
clamping techniques to better assess the extent of coupling
of ReCx45 cells among themselves and with ECs. In these studies,
each cell of a pair is voltage-clamped with an electrode,
and the cells are alternately stimulated with 0 to 40 mV. If
functional gap junctions exist between the 2 cells, current will
be detectable in the adjoining (nonstimulated) cell, and used to
calculate the macroscopic junctional conductance (gJ=recorded
current divided by transjunctional voltage difference; dia-
gram at the bottom of Figure 2), as described. This approach
revealed that 100% of ReCx45 pairs (n=8) were electrically
coupled. Mean macroscopic conductance of ReCx45 pairs
was 1.5±0.4 nS, which is consistent with Cx45-containing gap
junction channels (Figure 2B). When heterocellular pairs
were assessed (n=20), 50% of ReCx45–EC pairs were coupled, with
a mean conductance of 1.1±0.6 nS (Figure 2C). Thus, the com-
bined dye injection and electrophysiological (Figure 2) data
demonstrate that ReCx45 mesenchymal cells form functional
gap junctions among themselves and with ECs.

Cx45 Supports Endothelial-Induced
Mural Cell Differentiation
To determine whether Cx45 expression and formation of
functional heterocellular gap junctions supports endothelial-
duced mural cell differentiation, ReCx45 mesenchymal cells
(of either clone) were cocultured with ECs, and total protein
was isolated and analyzed for expression of mural cell-specific
markers. We observed an upregulation of smooth muscle (SM-
α-actin expression in ReCx45 mesenchymal cells on coculture
with ECs (versus total protein from mesenchymal cells alone;

Figure 3A and 3B), a response observed in Cx43wt, but lost in
Cx43−/−
mesenchymal cells (previous studies); Figure 3A and 3B). Consistent with previous studies,3 SM-α-actin was not detected in ECs (Figure 3B); therefore, its upregulation in endothelial–mesenchymal cocultures reflected changes in mesenchymal cell protein expression. Expression of other mural cell-specific markers (calponin, SM22α, and SM-myosin
heavy-chain) was also increased in ReCx45 cells, on coculture
with ECs (Figure 3C). Furthermore, we visualized expression
of mural cell-specific markers by immunocytochemistry.
Compared with solo cultures of ReCx45, expression of SM-α-
actin, SM-γ-actin, and SM-myosin heavy-chain was increased
in ReCx45-3 (and ReCx45-1; not shown) cells in coculture with
fluorescently labeled ECs, and was limited to mesenchymal
cells (Figure 3D). These data indicate that endothelial-induced
mural cell differentiation is restored by expression of Cx45 in
Cx43−/−
mesenchymal cells.

Coculture of ReCx45 Mesenchymal Cells
and ECs Enables TGF-β Activation
To determine whether Cx45-containing gap junction chan-
nels mediate TGF-β activation to enable mural cell differ-
entiation, activated TGF-β was measured in ReCx45 cells in
solo culture and in coculture with ECs. Activated TGF-β was
significantly elevated in ReCx45 and EC cocultures compared with solo cultures of either Cx43wt or ReCx45 mesenchymal cells (Figure 4A). Furthermore, as was previously shown for Cx43−/− and Cx43wt mesenchymal cells, exogenous TGF-β1 upregulated SM-α-actin expression in ReCx45 cells (Figure 4B). Collectively, these data demonstrate that heterocellular Cx45-containing gap junctions formed between mesenchymal cells and ECs promote the activation of TGF-β, which mediates endothelial-induced mural cell differentiation.

**Figure 3.** ReCx45 mesenchymal cells undergo mural cell differentiation in coculture with endothelial cell (EC). A, As previously found, expression of SM-α-actin was not upregulated in connexin (Cx) 43−/− mesenchymal cells cocultured with EC. However, its expression was elevated in ReCx45 mesenchymal cells cocultured with EC versus cultured alone. As described in Materials and Methods, twice the total protein is loaded in coculture lanes to permit comparison of SM marker expression from the mesenchymal cell fraction against total protein isolated from mesenchymal cell solo cultures. B, Densitometric analysis (n=3–6) revealed significant (*; Student t test) upregulation of SM-α-actin in cocultures of Cx43wt control (13-fold, inset) or ReCx45 (5-fold, inset) mesenchymal cells vs solo culture, which was not observed in cocultures of EC and Cx43−/− mesenchymal cells; additionally, no SM-α-actin expression was detected in total protein isolated from EC solo cultures. C, Upregulation of other mural cell-specific markers (calponin, SM22α, and SM-myosin heavy-chain [MHC]) was also observed in ReCx45 mesenchymal cells on coculture with EC. D, Increased expression of mural cell-specific markers (SM-α-actin, SM-γ-actin, and SM-MHC) in coculture of ReCx45 mesenchymal cells with PKH36-labeled EC (*) was limited to mesenchymal cells (scale bar=50 µm).

**Coculture of ReCx45 Mesenchymal Cells and ECs Upregulates Endothelial ECM Deposition Downstream of TGF-β Activation**

TGF-β activation depends, in part, on binding of latent TGF-β to the ECM, and subsequent cleavage of the TGF-β latency peptide by extracellular proteases. Therefore, it is possible that gap junction channel formation between ECs and mesenchymal cells promotes ECM production/deposition, and this, in turn, enables TGF-β activation. To test this, we first examined the expression of ECM proteins in solo cultures and cocultures of ECs and mesenchymal cells. We found that fibronectin (and fibrillin; not shown) was expressed by ECs (Figure 5A), but not by mesenchymal cells (Figure 5C), in solo culture, and persisted when ECs were cocultured with either Cx-expressing or Cx-deficient mesenchymal cells (Figure 5B). Therefore, gap junction channel formation was not required for ECM production/deposition in endothelial–mesenchymal cell cocultures.

Nonetheless, fibronectin (and fibrillin; not shown) deposition was upregulated in ECs cocultured with Cx43− or ReCx45-expressing mesenchymal cells, but not with Cx43− mesenchymal cells that do not form functional gap junctions with ECs (Figure 5B). The increased expression of ECM proteins in ECs cocultured with mesenchymal cells (Figure 5B) was similar to that observed when ECs were treated with exogenous TGF-β1 (Figure 5A), and neutralization of TGF-β in cocultures suppressed the upregulation of ECM proteins (Figure 5C). Furthermore, culturing Cx43− or ReCx45 mesenchymal cells onto fibronectin (Figure I in the online-only Data Supplement) did not promote upregulation of mural cell-specific proteins. Thus, ECM proteins are upregulated in endothelial–mesenchymal cocultures downstream of TGF-β activation, but ECM protein deposition alone does not appear to be sufficient to promote TGF-β activation and mural cell differentiation in the cocultures.

Collectively, these data demonstrate that heterocellular Cx45-containing gap junctions formed between mesenchymal cells and ECs promote TGF-β activation and enable endothelial–induced mural cell differentiation, in a process that is not dependent on the production/deposition of fibronectin or fibrillin. However, ECM proteins upregulated in response to gap junction-mediated TGF-β activation likely contribute to subsequent steps of blood vessel assembly/stabilization, including regulation of cell growth.

**Discussion**

We previously demonstrated that formation of gap junction channels is required for endothelial–mesenchymal cell
ECs and mesenchymal cells mediate these processes. In this study, we investigated whether Cx45 plays a similar role in the regulation of endothelial-induced mural cell differentiation. We found that Cx45 expression in Cx43-deficient mesenchymal cells restores formation of functional heterocellular gap junctions with ECs and enables TGF-β activation, presumably by stimulating release of an enzyme or activator that frees active TGF-β from its inactivating latency-associated peptide, thereby promoting the upregulation of mural cell-specific proteins in the mesenchymal cells. These findings are consistent with previous in vivo studies of Cx45-deficient embryos that exhibit dysregulated TGF-β signaling and lack mural cell development.

Still unclear, however, is why Cx43, which can support contact-induced TGF-β activation, which promotes mesenchymal cell differentiation toward a mural cell phenotype. We also showed that Cx43-containing gap junctions between ECs and mesenchymal cells mediate these processes. In this study, we investigated whether Cx45 plays a similar role in the regulation of endothelial-induced mural cell differentiation. We found that Cx45 expression in Cx43-deficient mesenchymal cells restores formation of functional heterocellular gap junctions with ECs and enables TGF-β activation, presumably by stimulating release of an enzyme or activator that frees active TGF-β from its inactivating latency-associated peptide, thereby promoting the upregulation of mural cell-specific proteins in the mesenchymal cells. These findings are consistent with previous in vivo studies of Cx45-deficient embryos that exhibit dysregulated TGF-β signaling and lack mural cell development.

Still unclear, however, is why Cx43, which can support these same processes and is frequently coexpressed with Cx45 in the embryonic and adult vasculature, does not compensate for lack of Cx45 at early stages of vascular development. This observation suggests that Cx45, specifically, regulates endothelial-induced mural cell differentiation during embryogenesis. This idea is further supported by the fact that Cx43-deficient embryos exhibit normal blood vessel formation and mural cell development during gestation, despite dying shortly after birth from severe defects in cardiovascular function. Thus, both Cx43 and Cx45 are required for normal vascular development, and may play similar roles in vivo, but at different stages of development and perhaps through different intracellular mechanisms.

Although we found that both Cx43- and Cx45-containing gap junctions formed between EC and mesenchymal cell precursors are capable of supporting TGF-β activation in heterocellular cocultures, it is not clear how these Cxs regulate this process. One possibility is that gap junction channel formation/function leads to upregulation of ECM proteins that are required for binding the latent TGF-β peptide complex for subsequent cleavage of the inhibitory peptide by extracellular proteases to activate this growth factor. However, we found that fibronectin and fibrillin, which have previously been associated with TGF-β activation in ECs and other cell types are present in endothelial–mesenchymal cell cocultures that do not form heterocellular gap junctions, yet TGF-β is not activated. These ECM proteins also failed to stimulate mural cell differentiation in ReCx45 or Cx43 mesenchymal cells that when cultured alone, we previously showed, express latent but not activated TGF-β, and also failed to restore mural cell differentiation in cocultures of Cx-deficient mesenchymal cells and ECs. Therefore, endothelial–mesenchymal heterocellular gap junction channel formation/function must regulate other intercellular signaling processes that are critical for TGF-β activation.

Historically, Cxs, as components of intercellular channels, were thought to mediate the intercellular exchange of soluble signals that regulate cellular processes and behaviors in a variety of cells, including vascular cells. However, Cxs can also exert channel-independent functions and stimulate, or respond to, intracellular signaling cascades via regulation (ie, phosphorylation) of their cytoplasmic regions. Thus, comparisons between the selective properties of Cx43 and Cx45 channels, as well as the regulatory regions of the proteins themselves, could provide clues as to how they mediate the activation of TGF-β and enable mural cell differentiation.

Interestingly, Cx45 and Cx43 form homomeric channels with notable differences in permeability and selectivity. Cx45 channels are cation-selective, whereas Cx43 channels are not charge-selective. In addition, Cx43 channels support higher permeability than Cx45 channels. Based on the selective properties of Cx45 versus Cx43 junctions, it appears that if a gap junction-traversing signal is required for TGF-β activation, then this signal may be a small positively charged molecule, perhaps calcium. Interestingly, intracellular calcium levels regulate many cellular processes, including secretion of enzymes known to activate TGF-β, such as cathepsin B, plasmin, thrombospondin, and metalloproteinase 2 and 9. Alternatively, if TGF-β activation requires dye (as well as ion)-permeable gap junction channels comprising
either Cx43 or Cx45, the communicated signal may be a larger signaling molecule, such as 1,4,5-inositol phosphate 3, a Ca\(^{2+}\)-activating signaling molecule important in TGF-\(\beta\)-signaling during mural cell differentiation.\(^{41}\) Clearly, additional studies are needed to determine whether exchange of a signaling molecule through functional channels is necessary and, if so, what the traversing signal might be.

The carboxyl-terminal domains of both Cx43\(^{42}\) and Cx45\(^{43,44}\) are targets for phosphorylation by a variety of kinases, and serve as binding sites for several intracellular proteins. The consequences of phosphorylation are multiple, and include acute and longer term regulation of channel function, as well as channel-independent protein–protein interactions. Comparison of the amino acid sequences of Cx45 and Cx43 carboxyl-terminal domains suggests, and functional studies confirm,\(^{43,45,46}\) that these proteins are likely targeted by some of the same, as well as distinct, protein kinases (Figure II and Table I in the online-only Data Supplement). For example, there are no high-probability consensus sites for mitogen-activated protein kinase-dependent phosphorylation in Cx45, but several in Cx43; in contrast, both Cxs share a serine-rich region at the ends of their C-terminal tails, containing several high-probability protein kinase C consensus sites (Cx43: S364, S368, and S372; Cx45: S378, S381, and S385). Because protein kinase C is central in transducing growth factor signals to their ultimate growth effects, and this kinase has been shown to regulate the dye and electrical permeability of Cx43,\(^{47}\) these conserved putative protein kinase C-targeted serines may underlie the requirement for Cx43 in TGF-\(\beta\) activation and endothelial-induced mural cell differentiation, and the ability of Cx45 to restore this response in the absence of Cx43.

As suggested above, TGF-\(\beta\) activation could also occur as a consequence of intracellular-signaling events initiated by the formation of either Cx43- or Cx45-comprised gap junctions, but independent of intercellular diffusion of soluble signals through open channels. As an example, during cardiomyocyte differentiation, Cx43 channel formation is required for and induces differentiation by competing with Smad2/3 for binding sites on \(\beta\)-tubulin.\(^{48}\) As Cx43 expression increases, Smad2/3 is released from its \(\beta\)-tubulin–binding site, phosphorylated (by activated activin-like kinase receptors), and translocated as a complex with SMAD4 to the nucleus, where it promotes expression of cardiomyocyte-specific genes. Relevant to our study, neither Cx45 nor Cx40 support cardiomyocyte differentiation in this manner because these proteins do not interact with tubulin at the Smad2/3-binding site. Thus, there may be Cx-specific differences in TGF-\(\beta\) activation and signaling, and channel function may not be necessary for these processes.

Clearly, dissecting the mechanism(s) by which Cx43- and Cx45-containing gap junctions mediate TGF-\(\beta\) activation and endothelial-induced mural cell differentiation will be complex and is beyond the scope of this study. However, ongoing studies are designed to address these issues and should shed light on the cellular and molecular regulation of these processes that are needed for blood vessel formation. Information gained from these studies will not only further our understanding of normal blood vessel development, but also provide needed insights into the regulation of neovascularization in postnatal tissues that occurs in response to tissue growth, injury, or progressive pathology.

**Sources of Funding**

These studies were supported by National Institutes of Health grants R01-HL077675 (to K.K.H. and J.M.B.) and R01-HL096360 (to K.K.H.).
Disclosures

None.

References


Connexin45 Regulates Endothelial-Induced Mesenchymal Cell Differentiation Toward a Mural Cell Phenotype

Jennifer S. Fang, Cuiping Dai, David T. Kurjiaka, Janis M. Burt and Karen K. Hirschi

*Arterioscler Thromb Vasc Biol.* 2013;33:362-368; originally published online December 6, 2012;
doi: 10.1161/ATVBAHA.112.255950

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/33/2/362

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/12/06/ATVBAHA.112.255950.DC1

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
http://atvb.ahajournals.org//subscriptions/
Supplement Material

Extended Materials and Methods

Cell Culture

Cx43-expressing (Cx43wt) and Cx43-deficient (Cx43<sup>−/−</sup>) mesenchymal cells, as well as Cx43<sup>−/−</sup> mesenchymal cells transfected with full-length Cx45 (ReC45), freshly isolated bovine aortic endothelial and smooth muscle cells were maintained as described. In some experiments, prior to cell plating, sterile coverslips were incubated for 24h in HBSS alone or treated with 0.25-1μg/cm<sup>2</sup> fibronectin (Sigma #F4759) at 37°C.

Generation of Cx45-Expressing Mesenchymal Cells

We obtained full-length mouse Cx45 cDNA in a pCl-neo vector (kindly provided by Dr. Klaus Willecke). To allow for selection of clones in a cell line expressing neomycin resistance, Cx45 cDNA was spliced into a pPUR vector (Clontech #631601) that contained the puromycin resistance gene. The Cx45-pPUR vector was stably transfected into mesenchymal cells isolated from E15.5 mice deficient for Cx43 (Cx43<sup>−/−</sup>, kindly provided by Dr. Alan Lau) using Lipofectamine (Invitrogen #11668). Individual clones were isolated from the stable transfectants (ReCx45) with 6 μg/ml puromycin (LG-DMEM and 10% FBS) and maintained in 2 μg/ml puromycin.

Cx45 Expression and Localization in Transfectants

Cx45-expressing cell lines were identified by Western blot and immunofluorescence. Total protein was isolated from all puromycin-resistant clones, and 10μg of protein was subjected to Western analysis using anti-Cx45 antibodies (Chemicon #MAB3100; 1:100), followed by HRP-linked secondary antibodies. Antibody-antigen complexes were revealed using ECL-Plus and documented with a digital imaging system. For immunocytochemistry, anti-Cx45 primary antibodies were used (Zymed #40-7000; 1:100) in conjunction with fluorescently-tagged
secondary antibodies (anti-Rabbit Alexa 594, Invitrogen #A21207; 1:100) to visualize intracellular expression and localization of Cx45 protein.

**Assessment of Gap Junction Communication**

To determine whether the expression of Cx45 enabled Cx43−/− mesenchymal cells to form functional gap junction channels amongst themselves and with endothelial cells, we employed the following techniques.

*Scrape Loading.* Cx43−/− or ReCx45 mesenchymal cells were plated into 6-well plates and allowed to form confluent monolayers. Upon confluence, cell medium was replaced with Ca2+-free PBS containing 40µg/mL Lucifer Yellow fluorescent dye (Molecular Probes #L-453) and 40µg/mL Rhodamine Dextran (Molecular Probes #D-1817). Cells were scraped using a modified straight razor to load wounded cells with both dyes, and incubated at 37°C for 15 minutes. Following incubation, cells were gently washed with Ca2+/Mg2+-containing PBS and immediately imaged. Wounded cells are marked with the gap junction-impermeable Rhodamine Dextran dye, whereas Lucifer Yellow permeates gap junction channels and marks adjacent cells that share functional gap junctions.24 For quantification, 3 random fields were imaged at the scrape edge, and the number of Lucifer Yellow-positive cells was compared to the number of Rhodamine Dextran-positive cells to determine the percentage of cells that exhibited gap junction dye coupling.

*Microinjection of Lucifer Yellow Dye.* (PKH26)-labeled3 endothelial cells were co-cultured 1:1 with ReCx45 mesenchymal cells onto glass coverslips. In heterocellular pairs, either cell type was microinjected with Lucifer Yellow (10% in 300 mmol/L LiCl) using glass micropipettes (Eppendorf). After 2 min, cells were photographed to document the presence or absence of dye in neighboring cells. Microinjections were performed on 10-20 cell pairs in cocultures in each of 4-5 separate experiments.3
**Dual Whole-Cell Voltage Clamp.** Fluorescently labeled (overnight in 50 μM Dil) endothelial and Cx45-expressing mesenchymal cells were co-plated onto glass coverslips, and homocellular and heterocellular pairs were subjected to dual whole-cell voltage clamp. Patch-type microelectrodes (~10 MΩ) were fabricated from 1.2 mm filament glass and back-filled with internal solution consisting of (in mmol/L): 135 KCl, 10 TEACl, 0.5 CaCl₂, 3 MgCl₂, 5 glucose, 10 HEPES, 10 EGTA, and 5 Na₂ATP (320 mOsm, pH 7.2). Cells were placed into an osmotically-balanced extracellular solution containing (in mmol/L): 142.5 NaCl, 4 KCl, 1 MgCl₂, 5 glucose, 2 sodium pyruvate, 10 HEPES, 1 BaCl₂, 1 CaCl₂, 15 CsCl, and 10 TEACl (320 mOsm, pH = 7.2). After voltage clamp was achieved, cells were alternately stepped between 0 and 10 mV, or 0 and 40 mV, to determine macroscopic junctional conductance (gj).

**Assessment of Endothelial-induced Mural Cell Differentiation**

To determine whether Cx45 expression in mesenchymal cells enabled their differentiation in response to endothelial cells, we assessed expression of smooth muscle (SM) cell specific proteins via Western blot analyses and immunocytochemistry. Endothelial and mesenchymal cells were cultured alone or in 1:1 co-culture for 48 hr in 2% FBS LG-DMEM. Thereafter, total protein was isolated; 1µg of protein from solo cultures and 2µg from co-cultures were subjected to Western analysis to evaluate the expression of SM-α-actin, SM-γ-actin, SM-MHC, SM22α, and calponin, as described. Antibody-antigen complexes were revealed using ECL-Plus and quantified using a digital imaging system. Densitometric analysis was performed using ImageJ software by normalizing band intensity of smooth muscle markers against either the total band intensity of the loading control for solo cultures, or against half the band intensity of the loading control for co-cultures. For immunocytochemistry, co-cultures of Cx45-expressing mesenchymal and PKH26-labeled (Sigma #PKH26-GL, cells labeled according to manufacturer protocol) endothelial cells were established in the Under Agarose system or via 1:1 simultaneous plating. After co-culture for 2 days, cells were fixed and immunostained for SM-α-actin, SM-myosin...
heavy chain, calponin, and SM22α. Antibody-antigen complexes were visualized using a high-sensitivity peroxidase kit (Vectastain #PK-6100) or fluorophore-conjugated secondary antibodies (Invitrogen #A21202).

**Measurement of Activated TGF-β**

An established bioassay was employed to measure levels of activated TGF-β. Mink lung epithelial cells (MLEC), expressing a truncated TGF-β targeted PAI-1 promoter linked to the luciferase reporter (kindly provided by Dr. Daniel Rifkin), were incubated in triplicate for 60 min with conditioned medium from mono- or co-cultures of endothelial and mesenchymal cells. Unconditioned medium containing 0 - 100 pg/ml TGF-β1 was similarly assayed to create a linear standard curve. Following exposure to conditioned or unconditioned media, MLEC were processed using a Luciferase Reporter Assay (Promega #E1500) and the mean amount of active TGF-β was calculated, as described.

**Treatment with Exogenous TGF-β1 or TGF-β Blocking Antibodies**

Cells were exposed for 48 hr to either 1ng/mL human TGF-β1 (R&D #100-B-001) or 10μg/mL TGF-β blocking antibodies that target all isoforms of TGF-β (Genzyme #1836-01) in low-glucose D-MEM supplemented with 2% FBS as previously described, prior to Western blot or immunohistochemical analysis.

**Assessment of Extracellular Matrix Deposition**

Solo cultures or co-cultures of PKH26-labeled endothelial cells and unlabeled mesenchymal cells were plated for 48 hr onto glass coverslips, and then fixed in 2% paraformaldehyde for 10 min and blocked in 3% BSA for 30 min. Coverslips were immunostained with pan-antibodies against fibronectin (BD #610077, 1:250) or fibrillin (Santa Cruz #sc-7540, 1:100). Antigen-antibody complexes were detected by incubation with fluorophore-conjugated antibodies (anti-
mouse (Invitrogen #A21202) or anti-goat (Invitrogen #A11055) Alexa488) prior to imaging. Quantification of ECM deposition was performed using ImageJ software. Five random fields from 4-12 experiments were imaged and the number of pixels above background in the ECM channel was averaged across each field for each slide.

Pair-wise Alignment and Kinase-Specific Prediction of Phosphorylation Sites

Peptide sequences for mouse Cx43 (Accession #P23242) and Cx45 (Accession #P28229) were downloaded from GenPept, and the predicted transmembrane regions listed for each Cx in their GenPept records were used to identify intracellular regions for each sequence. Pair-wise Clustal alignment was conducted using Clustal Omega multiple peptide alignment software available from the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/msa/clustalo/). Full-length peptide sequences for each Cx were also analyzed using kinase-specific phosphorylation site prediction software (NetPhosK 1.0, http://www.cbs.dtu.dk/services/NetPhosK/) with threshold set at 50%.

Statistics

Two-sample comparisons were made using a Students’ T-test. Multiple samples were compared by ANOVA analysis, followed by a Tukey-Kramer post-hoc T-test. In all cases, $\alpha$ was set to 0.05.
Supplemental Figure Legends

Supplemental Figure I: Fibronectin did not alter mesenchymal cell expression of SM-α-actin. Cx43−/− and ReCx45 mesenchymal cells were plated alone, or co-cultured with EC, on uncoated or fibronectin-coated coverslips and immunostained for SM-α-actin (green). The presence of fibronectin, alone, did not alter SM-α-actin expression in Cx43−/− and ReCx45 mesenchymal cells in the absence of EC. Moreover, fibronectin did not promote SM-α-actin expression in Cx43−/− mesenchymal cells even in the presence of EC. As expected, SM-α-actin was significantly increased in ReCx43 and ReCx45 in response to EC (scale bar = 20μm).

Supplemental Figure II: Cx43 and Cx45 amino acid sequences contain several conserved and non-conserved putative phosphorylation sites in cytoplasmic loop and C-terminal regulatory domains. Peptide sequences of mouse Cx45 and Cx43 were compared by pairwise Clustal Omega analysis, conserved residues are marked with an asterisk (*). Predicted transmembrane (TM) regions listed in the Genpept record for each gene are mapped in gray. Predicted kinase-specific phosphorylation sites detected in the intracellular loop and tail regions of each Cx with a 60% or greater score by NetPhosK 1.0 software26 (or for which experimental data46 demonstrate MAPK phosphorylation at the indicated sites, #) are marked. Complete NetPhosK 1.0 prediction results with a 50% or greater score are listed in Supplemental Table I.

Supplemental Table I: Phosphorylation sites predicted in rat Cx43 and Cx45 sequence by NetPhosK 1.0 software33 with a score above 50%. Acronyms: cdc2, cyclin-dependent kinase 1; cdk5, cyclin-dependent kinase 5; CKI, casein kinase I; CKII, casein kinase II; DNAPK, DNA-dependent protein kinase; EGFR, epidermal growth factor receptor; GSK3, glycogen synthase
kinase 3; INSR, insulin receptor; p38 MAPK, p38 mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C
### Supplemental Table I

#### Cx43

<table>
<thead>
<tr>
<th>Site</th>
<th>Kinase</th>
<th>Score (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y 17</td>
<td>EGFR</td>
<td>50</td>
</tr>
<tr>
<td>S 18</td>
<td>cdc2</td>
<td>54</td>
</tr>
<tr>
<td>T 19</td>
<td>PKC</td>
<td>58</td>
</tr>
<tr>
<td>S 27</td>
<td>cdc2</td>
<td>54</td>
</tr>
<tr>
<td>S 43</td>
<td>CKII</td>
<td>51</td>
</tr>
<tr>
<td>T 56</td>
<td>DNAPK</td>
<td>59</td>
</tr>
<tr>
<td>S 86</td>
<td>PKA</td>
<td>59</td>
</tr>
<tr>
<td>T 118</td>
<td>PKC</td>
<td>59</td>
</tr>
<tr>
<td>Y 137</td>
<td>INSR</td>
<td>55</td>
</tr>
<tr>
<td>S 180</td>
<td>PKA</td>
<td>60</td>
</tr>
<tr>
<td>T 186</td>
<td>PKC</td>
<td>87</td>
</tr>
<tr>
<td>S 217</td>
<td>PKA</td>
<td>52</td>
</tr>
<tr>
<td>S 220</td>
<td>PKA</td>
<td>57</td>
</tr>
<tr>
<td>Y 247</td>
<td>INSR</td>
<td>51</td>
</tr>
<tr>
<td>S 255</td>
<td>p38 MAPK</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>GSK3</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>cdk5</td>
<td>64</td>
</tr>
<tr>
<td>S 257</td>
<td>cdc2</td>
<td>52</td>
</tr>
<tr>
<td>S 262</td>
<td>p38 MAPK</td>
<td>52</td>
</tr>
<tr>
<td>S 272</td>
<td>cdc2</td>
<td>50</td>
</tr>
<tr>
<td>S 273</td>
<td>p38 MAPK</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>cdk5</td>
<td>51</td>
</tr>
<tr>
<td>S 279</td>
<td>p38 MAPK</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>GSK3</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>cdk5</td>
<td>70</td>
</tr>
<tr>
<td>S 282</td>
<td>p38 MAPK</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>GSK3</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>cdk5</td>
<td>63</td>
</tr>
<tr>
<td>T 290</td>
<td>PKC</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>cdc2</td>
<td>53</td>
</tr>
<tr>
<td>S 297</td>
<td>PKC</td>
<td>53</td>
</tr>
<tr>
<td>S 325</td>
<td>PKC</td>
<td>87</td>
</tr>
<tr>
<td></td>
<td>cdc2</td>
<td>57</td>
</tr>
<tr>
<td>T 326</td>
<td>cdc2</td>
<td>55</td>
</tr>
<tr>
<td>S 364</td>
<td>PKC</td>
<td>82</td>
</tr>
<tr>
<td>S 365</td>
<td>cdc2</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>GSK3</td>
<td>50</td>
</tr>
<tr>
<td>S 368</td>
<td>PKC</td>
<td>86</td>
</tr>
<tr>
<td>S 369</td>
<td>RSK</td>
<td>50</td>
</tr>
<tr>
<td>S 372</td>
<td>PKC</td>
<td>89</td>
</tr>
<tr>
<td>S 373</td>
<td>cdc2</td>
<td>54</td>
</tr>
</tbody>
</table>

#### Cx45

<table>
<thead>
<tr>
<th>Site</th>
<th>Kinase</th>
<th>Score (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S 2</td>
<td>PKA</td>
<td>54</td>
</tr>
<tr>
<td>T 18</td>
<td>PKC</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>cdc2</td>
<td>54</td>
</tr>
<tr>
<td>T 26</td>
<td>PKC</td>
<td>50</td>
</tr>
<tr>
<td>S 42</td>
<td>CKII</td>
<td>51</td>
</tr>
<tr>
<td>Y 44</td>
<td>SRC</td>
<td>54</td>
</tr>
<tr>
<td>S 72</td>
<td>PKC</td>
<td>60</td>
</tr>
<tr>
<td>S 114</td>
<td>PKC</td>
<td>72</td>
</tr>
<tr>
<td>T 130</td>
<td>CKII</td>
<td>69</td>
</tr>
<tr>
<td>S 148</td>
<td>CKII</td>
<td>69</td>
</tr>
<tr>
<td>S 156</td>
<td>DNAPK</td>
<td>63</td>
</tr>
<tr>
<td>S 210</td>
<td>PKG</td>
<td>55</td>
</tr>
<tr>
<td>T 226</td>
<td>PKC</td>
<td>59</td>
</tr>
<tr>
<td>S 264</td>
<td>PKC</td>
<td>86</td>
</tr>
<tr>
<td>Y 275</td>
<td>EGFR</td>
<td>51</td>
</tr>
<tr>
<td>T 283</td>
<td>cdk5</td>
<td>64</td>
</tr>
<tr>
<td>S 285</td>
<td>PKG</td>
<td>53</td>
</tr>
<tr>
<td>Y 290</td>
<td>EGFR</td>
<td>51</td>
</tr>
<tr>
<td>Y 324</td>
<td>SRC</td>
<td>53</td>
</tr>
<tr>
<td>S 326</td>
<td>CKII</td>
<td>58</td>
</tr>
<tr>
<td>S 374</td>
<td>cdc2</td>
<td>50</td>
</tr>
<tr>
<td>Y 356</td>
<td>EGFR</td>
<td>51</td>
</tr>
<tr>
<td>S 378</td>
<td>cdc2</td>
<td>53</td>
</tr>
<tr>
<td>S 381</td>
<td>PKC</td>
<td>78</td>
</tr>
<tr>
<td>S 381</td>
<td>PKC</td>
<td>70</td>
</tr>
<tr>
<td>S 382</td>
<td>PKC</td>
<td>50</td>
</tr>
<tr>
<td>S 384</td>
<td>cdc2</td>
<td>51</td>
</tr>
<tr>
<td>S 385</td>
<td>CK1</td>
<td>56</td>
</tr>
<tr>
<td>S 387</td>
<td>PKC</td>
<td>68</td>
</tr>
<tr>
<td>S 387</td>
<td>PKC</td>
<td>57</td>
</tr>
</tbody>
</table>