MAPK Phosphorylation of Connexin 43 Promotes Binding of Cyclin E and Smooth Muscle Cell Proliferation


Rationale: Dedifferentiation of vascular smooth muscle cells (VSMC) leading to a proliferative cell phenotype significantly contributes to the development of atherosclerosis. Mitogen-activated protein kinase (MAPK) phosphorylation of proteins including connexin 43 (Cx43) has been associated with VSMC proliferation in atherosclerosis.

Objective: To investigate whether MAPK phosphorylation of Cx43 is directly involved in VSMC proliferation.

Methods and Results: We show in vivo that MAPK-phosphorylated Cx43 forms complexes with the cell cycle control proteins cyclin E and cyclin-dependent kinase 2 (CDK2) in carotids of apolipoprotein-E receptor null (ApoE−/−) mice and in C57BI/6 mice treated with platelet-derived growth factor–BB (PDGF). We tested the involvement of Cx43 MAPK phosphorylation in vitro using constructs for full-length Cx43 (Cx43) or the Cx43 C-terminus (Cx43CT) and produced null phosphorylation Ser>Ala (Cx43MK4A/Cx43CTMK4A) and phosphomimetic Ser>Asp (Cx43MK4D/Cx43CTMK4D) mutations. Coimmunoprecipitation studies in primary VSMC isolated from Cx43 wild-type (Cx43+/+) and Cx43 null (Cx43−/−) mice and analytic size exclusion studies of purified proteins identify that interactions between cyclin E and Cx43 requires Cx43 MAPK phosphorylation. Finally, using a novel knock-in mouse containing Cx43-MK4A mutation, we show in vivo that interactions between Cx43 and cyclin E are lost and VSMC proliferation does not occur after treatment of carotids with PDGF and that neointima formation is significantly reduced in carotids after injury.

Conclusions: We identify MAPK-phosphorylated Cx43 as a novel interacting partner of cyclin E in VSMC and show that this interaction is critical for VSMC proliferation. This novel interaction may be important in the development of atherosclerotic lesions. (Circ Res. 2012;111:201-211.)

Key Words: connexin 43  ■  phosphorylation  ■  atherogenesis  ■  platelet-derived growth factor  ■  vascular smooth muscle cells  ■  proliferation  ■  neointima

Vascular smooth muscle cell (VSMC) proliferation is a major component of disease progression in atherosclerosis and in its treatment, for example, after angioplasty and in restenosis.1–3 In the earliest stages of atherosclerosis (atherogenesis) VSMC dedifferentiate in response to atherogenic stimuli, for example, oxidized phospholipids and growth factors, such as platelet-derived growth factor–BB (PDGF).4,5 Dedifferentiated VSMC increase atherosclerotic plaque size by proliferating and migrating into the plaque, followed by foam cell formation and senescence and through ongoing cellular proliferation within the plaque.6,7 Treatment of advanced plaques through angioplasty produces the unwanted side effect of stimulating further VSMC proliferation, leading to neointima formation and significantly reducing the vessel lumen.8,9 Advanced plaques are composed of a number of different cell types including macrophages and foam cells derived from both monocytes and from VSMC originating in the medial layers of arterial vessels.9–11 Therefore, identifying the causes of VSMC proliferation becomes central in the control of atherosclerosis and its treatments.
and VSMC proliferation in particular Cx43, which has a role in cellular proliferation. Connexins typically form gap junctions through a dodecameric association of connexin proteins that produces a functional channel between 2 cells that allows for coordinated cellular responses. Changes in the expression levels of Cx43 have been associated with VSMC proliferative potential in atherogenesis, in atherosclerotic plaque development and in restenosis. In addition, multiple studies have now demonstrated that Cx43 can modulate cellular proliferation in a manner that is independent of gap junctional communication. However, the exact pathways through which Cx43 modulates cellular proliferation are not known.

Activation of mitogen-activated protein kinase (MAPK) pathways promotes VSMC proliferation in atherogenesis. Many proatherogenic agents (eg, PDGF) have been shown to activate MAPK pathways, yet the mechanisms through which these pathways induce VSMC proliferation are poorly defined. Several lines of evidence indicate that MAPK phosphorylation of Cx43 can occur at specific C-terminus (CT) residues, for example, S255/S262/S279/282, and occurs differentially throughout the cell cycle. Recently, we identified that Cx43 becomes phosphorylated at its MAPK serines in response to atherogenic stimuli and that this is associated with VSMC proliferation both in vivo and in vitro. We demonstrate from the single protein level that this is associated with VSMC proliferation both in vivo and in vitro.

**Methods**

An expanded Methods section is available in the Online Data Supplement.

**Mice**

C57/B16 (10–18 weeks), ApoE<sup>−/−</sup> (15–18 weeks), and novel Cx43-MK4A (10–18 weeks) mice were used according to the University of Virginia Animal Care and Use Committee guidelines.

**Results**

**Cx43 Colocalizes With Cyclin E in VSMC and In Vivo**

To test whether Cx43 protein expression and its posttranslational modification are linked to VSMC proliferation, we initially screened potential binding partners of Cx43 that are associated with the G1 phase of the cell cycle including cyclin E, cyclin D1, p21<sup>Waf1/cip1</sup>, and p27<sup>Kip1</sup>. Coimmunoprecipitation studies identified that Cx43 associated with cyclin E but not the other proteins tested (Online Figure V). We therefore tested the ability of Cx43 to interact with cyclin E in vivo by immunofluorescence on carotid vessel sections. We identified marked increases in protein expression for both Cx43 and cyclin E within the carotid VSMC after PDGF treatment as compared with control conditions (Figure 1A). Using the proximity ligation assay, we further demonstrated almost no detectable interactions between Cx43 and cyclin E under control conditions but significant increases in interaction after PDGF treatments (Figure 1B). To determine the spatial interaction between the proteins in vivo we analyzed colocalization between Cx43, cyclin E, and CDK2 by immunofluorescence on carotid vessel sections. We identified marked increases in protein expression for both Cx43 and cyclin E within the carotid VSMC [ie, large average distance between beads of 490±70 nm (Cx43-cyclin E) and 379±72 nm (Cx43-CDK2; Figure 1D) were observed]. In VSMC of carotids from PDGF-treated mice and in ApoE<sup>−/−</sup> mice containing proliferative VSMC, significant reductions in distances between Cx43-cyclin E, Cx43-CDK2, and cyclin E–Cx43-CDK2 were identified with distance averaging 10 to 20 nm, indicative of interaction between the proteins (Figure 1D). Under control conditions, Cx43 MAPK phosphorylation was not readily detectable, with too few beads present as to be quantified on all sections (Figure 1E). However, treatments with PDGF and in ApoE<sup>−/−</sup> mice (as described) increased Cx43 MAPK phosphorylation, and interactions were identified with cyclin E and CDK2 (Figure 1E).

**Electron Microscopy**

Carotid arteries were imaged for VSMC morphology and immuno-labeled using Cx43, cyclin E, or CDK2 antibodies and detected with 25/15/10 nm gold beads, respectively, and imaged using a JEOL 6400 scanning electron microscope.

**Plasmids and Mutagenesis**

Derivatives of pCDNA3.1 and pGEX-6P-2 for expression of full length Cx43 (Cx43, a.a.1–382, pCDNA3.1), the Cx43 C-terminus (Cx43<sup>CT</sup>), rat, a.a. 236–382, pCDNA3.1, and pGEX-6P-2), and for full-length human cyclin E (pGEX-6P-2) were used. Mutagenesis of Cx43 MAPK serines to either alanines or aspartates was performed by Quickchange (Strategene). Plasmids were used for in vitro transfection or analysis of protein interaction by analytic size exclusion chromatography.

**Statistical Analysis**

Student t test, 1-way ANOVA, or 2-way ANOVA, followed by Bonferroni posttest, were used for comparisons between treatments. A probability value of <0.05 was significant.
(Cx43+/+; Online Figure II). Increases in Cx43, cyclin E, and CDK2 protein expression and coimmunoprecipitation of Cx43 with cyclin E and CDK2 proteins were identified after 24-hour treatments with PDGF (Online Figure VI). To define the cellular compartment of protein interaction and to identify the temporal relationship between Cx43 and cyclin E interactions, we investigated protein expression levels and coimmunoprecipitation by Western blot in total protein lysates and membrane fractions of Cx43+/+ VSMC. After PDGF treatments, expression of Cx43, MAPK-phosphorylated Cx43 (Cx43-P), and cyclin E all increased after 6 hours of treatment with PDGF in total protein lysates and membrane fractions of Cx43+/+ VSMC. After PDGF treatments, expression of Cx43, MAPK-phosphorylated Cx43 (Cx43-P), and cyclin E all increased after 6 hours of treatment with PDGF in total protein lysates and with increases in CDK2 observed from 12 hours (Figure 2A). In membrane fractions, expression of Cx43, Cx43-P, and cyclin E increased between 6- to 12-hour time points, after which marked reductions were observed (Figure 2A). Coimmunoprecipitation studies demonstrated that Cx43 and cyclin E form in complex by 12 hours in membrane fractions and were detectable at both 12 and 24 hours in total protein lysates (Figure 2A). To determine the effects on downstream targets of cyclin E and CDK2 activation, we performed Western blots of retinoblastoma (Rb) protein expression and its phosphorylation (pRB).

After PDGF treatments, we identified in Rb protein expression, and phosphorylation (pRb-780 and pRb-807/811) was increased between 12 to 24 hours after PDGF treatments (Figure 2B). To define whether MAPK phosphorylation of Cx43 was critical in this interaction, Cx43+/+ VSMC grown in low serum media were pretreated with extracellular signal-regulated protein kinase (ERK) inhibitors U0126 or Roscovitine followed by PDGF treatment. Both U0126 and Roscovitine inhibited the ability of Cx43 to become phosphorylated at MAPK serines and inhibited interactions with cyclin E and corresponded to significant reductions in VSMC proliferation (Figure 2C). Using Cx43+/+ cells treated with PDGF in the presence of U0126 or Roscovitine, we saw significant reductions in VSMC proliferation, indicating that MAPK phosphorylation of Cx43 is important in the proliferative phenotype of VSMC in response to PDGF (Figure 2C).

**Cx43 Interactions With Cyclin E Are Controlled Through MAPK Phosphorylation**

To further examine the role of Cx43 MAPK phosphorylation, VSMC were isolated from aortas of newborn Cx43+/+,
Cx43+/−, or Cx43−/− mice (Figure 3A), and we generated plasmids with mutations at the 4 C-terminus MAPK serines (S255/262/279/282) to mimic constitutive phosphorylation (Cx43MK4D) or null phosphorylation (Cx43MK4A) (Figure 3B and Online Figure IV). These plasmids were transfected into Cx43+/− VSMC at nearly equivalent levels (Figure 3C). Dye transfer studies in control and PDGF-treated VSMC transfected with the different plasmids showed no significant differences in gap junctional communication (Online Figure VII). Pretreatment of Cx43+/− VSMC grown in low serum, pretreated with ERK inhibitors (U0126 and Roscovitine) or DMSO, then treated with PDGF for 24 hours were analyzed by Western blot with antibodies against Cx43, Cx43-P, cyclin E, CDK2 and by coimmunoprecipitation with cyclin E–coated beads and detection of Cx43 by Western blot (C, top). VSMC proliferation of Cx43+/− VSMC grown in low serum, pretreated with ERK inhibitors (U0126 and Roscovitine) or DMSO, then treated with PDGF for 24 hours was assayed by flow cytometry (C, bottom). In A through C, black arrowheads identify the expected molecular weight for each protein; colored arrowheads correspond to molecular weights: green is 25 kDa, blue is 37 kDa, red is 50 kDa, yellow is 100 kDa. In C, triple asterisk indicates P<0.001, n=4.

Figure 2. In vitro identification of Cx43 colocalization with cell cycle proteins. Total protein and membrane fraction lysates from Cx43+/− VSMC grown in low serum and treated with PDGF harvested at specific time points over a 24-hour time course. Samples were analyzed by Western blot with antibodies against Cx43, Cx43-P, cyclin E, CDK2, GAPDH, or β-tubulin and by coimmunoprecipitation with cyclin E–coated beads and detection of Cx43 by Western blot (A). Specificity of Western blot detection is shown using antibody-coated beads treated with lysis buffer only to eliminate IgG contamination (A). Expression of Rb, phosphorylated retinoblastoma (pRb)-780, and pRb-807/811 were detected in total protein and membrane fraction lysates from PDGF time course experiments (B). Total protein lysates from Cx43+/− VSMC grown in low serum, pretreated with ERK inhibitors (U0126 and Roscovitine) or DMSO, then treated with PDGF for 24 hours were analyzed by Western blot with antibodies against Cx43, Cx43-P, cyclin E, or CDK2 and by coimmunoprecipitation with cyclin E–coated beads and detection of Cx43 by Western blot (C, top). VSMC proliferation of Cx43+/− VSMC grown in low serum, pretreated with ERK inhibitors (U0126 and Roscovitine) or DMSO, then treated with PDGF for 24 hours was assayed by flow cytometry (C, bottom). In A through C, black arrowheads identify the expected molecular weight for each protein; colored arrowheads correspond to molecular weights: green is 25 kDa, blue is 37 kDa, red is 50 kDa, yellow is 100 kDa. In C, triple asterisk indicates P<0.001, n=4.

To show that Cx43 C-terminus specifically binds cyclin E, we generated and used purified Cx43 C-terminus and glutathione S-transferase (GST)-tagged cyclin E proteins and evaluated complex formation using analytical size exclusion chromatography (Figure 3E and 3F). Individually, Cx43 C-terminus proteins (≈17 kDa; monomer) eluted in the same fractions (16–19) and cyclin E (≈75 kDa) eluted in fractions 15 to 17 (Figure 3F). In all experiments, we did not detect significant alterations in either the Cx43CT or the Cx43CTMK4A proteins when combined with cyclin E (Figure 3F). However, after incubation of Cx43CTMK4D proteins with cyclin E, we identified a marked shift in both Cx43CTMK4D and cyclin E. Both proteins coeluted earlier in the elution profile (fractions 14–17; increase in size), indicating complex formation of Cx43CTMK4D with cyclin E (Figure 3F). By cross-linking proteins in solution of fraction 15 from Cx43CTMK4D with cyclin E samples, we identified high-molecular-weight forms of both Cx43 C-terminus and cyclin E at ≈110 kDa by Western blot, corresponding to a stoichiometry of a single cyclin E and Cx43CTMK4D in dimer form (≈109 kDa; Figure 3G).
Based on the observations using purified proteins, we aimed to determine whether expressing the free Cx43 C-terminus in VSMC would lead to binding of cyclin E and alterations in cellular proliferation. We therefore transfected Cx43/Cx43/Cx43 VSMC with plasmids to express Cx43CT, Cx43CTMK4A, and Cx43CTMK4D proteins (Online Figure VIII). All 3 proteins were found to be expressed in the transfected VSMC but did not form interactions with cyclin E and did not confer increased VSMC proliferation in response to PDGF treatment (Online Figure VIII). We further determined that while all Cx43 C-terminus proteins were expressed, they did not traffic to the cellular membranes unlike full length Cx43 proteins when transfected to Cx43−/− VSMC (Online Figure VIII).

Figure 3. Cx43 interactions with cyclin E are dependent on MAPK phosphorylation in vitro. The expression of vascular connexins Cx43, Cx37, and Cx45 in VSMC isolated from newborn mouse aortas was identified by Western blotting (A). Site-directed mutagenesis was performed for the MAPK sites in both full-length Cx43 (a.a.1–382) and Cx43CT (a.a. 236–382) for phosphomimetic (Aspartate, Cx43MK4D, Cx43CTMK4D) and null phosphorylation (Cx43MK4A, Cx43CTMK4A) constructs (B). Primary VSMC from Cx43−/− mice were transfected with Cx43 plasmids and expression confirmed by Western blotting of Cx43 (C). Levels of expression were quantified against loading for GAPDH (n=3, C). Lysates from Cx43−/− VSMC transfected with each of the Cx43 plasmids and treated with PDGF were incubated with cyclin E–coated beads, then protein detection performed by Western blot analysis of Cx43 (n=3, D). Cx43CT-GST and cyclin E1–GST proteins were purified by GST bead purification followed by cleavage (Cx43CT proteins only) or elution (glutathione, cyclin E only). Samples were further purified to >90% purity by size exclusion chromatography (SEC) with Cx43CT detected at approximately 17 kDa and cyclin E at 75 kDa due to the addition of the 25-kDa GST tag (E). Purified proteins for Cx43CT, Cx43CTMK4A, Cx43CTMK4D, and cyclin E were assessed for in vitro binding via analytical size exclusion chromatography either as solo proteins or in combination (Cx43CT + cyclin E). Eluted fractions were analyzed for the expression of Cx43 (Cx43) or cyclin E (polyclonal) by Western blotting (n=2, F). After analytical size exclusion chromatography analysis, samples from elution fraction 15 from the Cx43CTMK4D + cyclin E samples were cross-linked using BS3 followed by Western blot analysis (G). In C through E, black arrowheads identify the expected molecular weight for each protein; colored arrowheads correspond to molecular weights: green is 25 kDa, blue is 37 kDa, red is 50 kDa, yellow is 100 kDa.

VSMC Proliferation Is Controlled Through MAPK Phosphorylation of Cx43
To identify a potential functional consequence of Cx43-cyclin E interaction, we compared proliferation in Cx43+/+, Cx43−/−, and Cx43−/− VSMC grown in low serum media in response to PDGF stimulation. Flow cytometric analysis indicated that low serum induced a cell cycle stall (approximately 5% proliferation) in all cells. However, loss or reductions of Cx43 (Cx43−/−, Cx43+/−, respectively) significantly ablates PDGF-induced VSMC proliferation as compared with Cx43+/+ cells (Figure 4A). Western blot analysis of cyclin E and CDK2 expression in Cx43−/− VSMC demonstrate that PDGF does not induce marked increases in cyclin E expression; however,
CDK2 protein expression was increased by 24 hours in the absence of Cx43 expression (Online Figure IX). Transfection of Cx43<sup>−/−</sup> VSMC with each of the Cx43 plasmids followed by treatment with PDGF produced a restoration of cell proliferation in cells expressing both Cx43 and the Cx43MK4D proteins but not the Cx43<sup>MK4A</sup> protein (Figure 4B).

**Loss of MAPK Phosphorylation of Cx43 In Vivo Reduces Interactions With Cyclin E and VSMC Proliferation**

To investigate the in vivo effects of Cx43 phosphorylation on VSMC proliferation in vivo, we generated a novel knock-in mouse line containing alanine mutation for Cx43 MAPK sites (Cx43-MK4A; Online Figure I) that displays similar levels of Cx43 expression to C57Bl/6 mice (Figure 5A). Analysis by transmission electron microscopy (TEM) and through expression of SM-actin by immunofluorescence revealed that PDGF treatment did not produce any apparent phenotypic alterations in the carotid VSMC of Cx43-MK4A mice, in contrast to those that were identifiable in C57Bl/6 carotid VSMC (Figure 5B and Online Figure X). Analysis of protein colocalization in Cx43-MK4A mice in vivo using i-TEM and immunofluorescence identified a loss of interaction between Cx43 and cyclin E or CDK2 in PDGF-treated carotid VSMC of Cx43-MK4A mice (Figure 5C and Online Figure XI). Treatment of C57Bl/6 mouse carotid VSMC with PDGF produced significant increases in VSMC proliferation (20.4±4.4%), but there was no evidence of VSMC proliferation in Cx43-MK4A mice treated with PDGF (Figure 5D).

**Loss of MAPK Phosphorylation of Cx43 In Vivo Reduces Neointima Formation**

To determine whether phosphorylation of Cx43 plays a significant role in neointimal formation, we performed carotid ligation (injury) assays that have been previously demonstrated to produce neointima formation in wild-type mice. Carotids from ligated C57Bl/6 mice demonstrated significant development of neointima, reduced luminal diameter, and reductions in expression of SM-actin in media VSMC that was not identified in Cx43-MK4A mice (Figure 6A through 6C and Online Figure XII). Increases in both Cx43 and MAPK-phosphorylated Cx43 were identified in both the media and neointima layers of C57Bl/6 mice but not within in the media layers of Cx43-MK4A mice (Figure 6D and 6E).

**Discussion**

A common factor in the promotion of VSMC proliferation is phosphorylation of proteins through activation of MAPK pathways. We recently identified that MAPK phosphorylation of Cx43 correlates to VSMC proliferation in ApoE<sup>−/−</sup> mice. In the present study, we identify that in response to atherogenic stimuli, that is, PDGF and vascular injury, for example, carotid ligation, Cx43 is phosphorylated at its C-terminus MAPK residues (S255/S262/S279/S282) and that this is a key regulator of VSMC proliferation and neointima formation in vivo. We further demonstrate that the mechanism underlying the proliferative response depends on a direct interaction of the Cx43 C-terminus with the cell cycle control protein cyclin E. These results are summarized in Online Figure XII.

Vascular smooth muscle cell proliferation in atherogenesis has been linked with an increase in the expression of PDGF. It has also been shown that PDGF stimulation increases the expression of cyclin E, cyclin D1, and their associated kinases (ie, CDK2 and CDK4), all of which are positive regulators of cellular proliferation. In addition, PDGF alters the expression levels of Cx43 which has been linked to enhanced VSMC proliferation. In keeping with these studies, we identified increases in Cx43, cyclin E, and cyclin D1 in VSMC in response to PDGF treatments in vitro. Using proliferative VSMC (10% serum), we found that Cx43 forms interactions with cyclin E but not with cyclin D1 or the cell cycle inhibitors p21<sup>wafl/cip1</sup> or p27<sup>kip1</sup>. Treatment of VSMC with PDGF induced MAPK phosphorylation of Cx43 and promoted formation of Cx43 complexes with cyclin E and its associated kinase CDK2. Complexes between MAPK-phosphorylated Cx43 with cyclin E and CDK2 were also identified in PDGF-treated C57Bl/6 and in ApoE<sup>−/−</sup> mice, as demonstrated through proximity ligation and i-TEM in vivo. These data indicate that Cx43 may act to interact with specific cell cycle proteins in proliferative VSMC.
Whereas cyclin E is ubiquitously expressed in cells, formation of active complexes with CDK2 occur at the cell membrane and is followed by removal of the complex from the cell membrane. Accumulation of cyclin E-CDK2 complexes in s-phase of the cell cycle can be further regulated by MAPK activation. In our studies, we identified that PDGF temporally increases global expression of Cx43, Cx43-P, cyclin E, and CDK2 proteins over a 24-hour period and that Cx43 and cyclin E protein expression increased transiently in membrane fractions with marked reductions found between 12 to 24 hours. We further show that Cx43-cyclin E complexes were present at 12 hours in membrane fractions and in total protein isolates between 12 to 24 hours. Increases in Cx43-cyclin E binding corresponded to an increase in the expression and phosphorylation of Rb, a downstream target of the cyclin E-CDK2 complex that acts to promote cellular proliferation. Activation of MAPK pathways, for example, ERK1/2, after increased PDGF expression promotes a proliferative phenotype in VSMC. After treatments with known ERK pathway inhibitors, for example, U0126 and CDK2 inhibitor roscovitine, we show that interactions between Cx43 and cyclin E can be inhibited and VSMC proliferation significantly reduced. Expression of free Cx43CT proteins within Cx43−/− VSMC cells failed to produce an interaction...
with cyclin E and failed to elicit PDGF induced proliferation as can be seen for the full-length protein. In keeping with previous reports that expression of the free Cx43CT leads to a diffuse pattern of staining within cells and does not affect cellular proliferation,39 we further show that the free Cx43CT does not target the cell membrane, suggesting that membrane localization is a key component in the interaction between Cx43 and cyclin E. However there are previous reports that the free Cx43<sup>CT</sup> protein expressed in HeLa cells can inhibit cellular proliferation although the pathways associated with this have not been clearly demonstrated.40 These data indicate that PDGF induces prolifer-

Figure 6. MAPK phosphorylation is required for neointima formation after carotid injury. Cross sections from C57Bl/6 and Cx43-MK4A mice under control or injury conditions were stained by hematoxylin and eosin for analysis of neointima formation (A). Expression of smooth muscle actin (SM22-α, magenta) was detected in media and neointima layers by immunofluorescence (B). Measures for the areas corresponding to neointima and media layers were used to calculate the ratio in control and injured C57Bl/6 and Cx43-MK4A mice (C, n=7). Immunofluorescent detection of Cx43 (D) and Cx43-P (E) in control and injured C57Bl/6 and Cx43-MK4A mice is shown. Scale bars in A, B, D, and E are 50 μm. In C, asterisk indicates P<0.05; double asterisk indicates P<0.01.
vation in VSMC through a mechanism that involves binding of MAPK-phosphorylated Cx43 to cyclin E and that formation of a complex between Cx43 and cyclin E initiates at the membrane and is followed by an internalization of the complex which promotes retinoblastoma phosphorylation and cell cycle progression.

It has previously been shown that PDGF increases Cx43 expression levels but that PDGF-induced MAPK phosphorylation of Cx43 reduces gap junction permeability leading to the hypothesis that gap junctional signaling pathways may contribute regulation of VSMC proliferation.41–43 Similar studies identified that truncation of Cx43 C-terminus (a.a. 256) significantly reduces the PDGF-induced response, potentially indicating that gap junctional communication is involved but also suggest that there may also be a connexin mediated (but non–gap junctional) component to PDGF-associated cell cycle regulation.44,45 In agreement with these studies, we find that a loss or reduction of Cx43 expression in Cx43−/− and Cx43+/− VSMC (respectively) correlates to significant reductions in the PDGF-induced proliferative response. We also demonstrate a rescue of the proliferative response in Cx43−/− VSMC transfected to express Cx43 and Cx43MK4D. Despite this, levels of gap junction communication were not significantly altered in the Cx43−/− VSMC as compared with Cx43+/+ VSMC. A reduction in dye transfer was observed in Cx43−/− after treatment with CBX indicating that gap junctional communication does occur within Cx43−/− VSMC. The identification of dye transfer in Cx43−/− cells suggests that they are still functionally coupled potentially through the remaining Cx37 and Cx45 identified within the cells. However, treatments with CBX failed to significantly reduce VSMC proliferation suggesting that proliferation can occur in a manner that is independent of gap junctional communication. Additionally, when Cx43−/− VSMC were transfected to express Cx43, the null phosphorylation (Cx43MK4A) or the phospho-mimetic (Cx43MK4D) forms, gap junctional communication was not significantly altered. However, in Cx43−/− VSMC transfected to express Cx43MK4A, the interaction between Cx43 and cyclin E was lost and there was no return to proliferation. Conversely, Cx43−/− VSMC expressing Cx43 and Cx43MK4D demonstrate formation of Cx43-cyclin E complexes and a return to proliferation in response to PDGF. Taken together, these data suggest that in response to PDGF, Cx43 becomes phosphorylated at its MAPK sites and interacts with cyclin E, which, in turn, promotes VSMC proliferation in a manner that is independent of gap junctional communication. Despite this, PDGF treatments did not significantly reduce gap junctional communication in our cells, and this leaves the possibility that a role for gap junctions does exist in these pathways.

To confirm our findings, we generated a novel knock-in mouse (Cx43-MK4A) that contains the Cx43 MAPK serine to alanine substitution expressed on the endogenous promoter for Cx43. In the Cx43-MK4A mice, we found levels of Cx43 expressed in carotid VSMC were similar to C57Bl/6 mice and showed that under control and PDGF-treated conditions there was no evidence for Cx43 MAPK phosphorylation in the Cx43-MK4A mice. Although Cx43 was present in the VSMC as well as cyclin E and CDK2, we could not identify interactions between Cx43 and cyclin E or Cx43 and CDK2. This finding is consistent with a requirement for Cx43 MAPK phosphorylation in forming interactions with cyclin E. In addition, we demonstrated a lack of VSMC proliferation in Cx43-MK4A mice and did not observe phenotypic changes in VSMC as identified in C57Bl/6 mice in response to PDGF treatment. In response to vascular injury, VSMC dedifferentiate and migrate to the intima of the vessel producing the neointima.28,46–47 Previous studies have demonstrated that Cx43 expression is significantly enhanced in the medial and neointimal VSMC after vascular injury.14–16,48 Development of neointima has been associated to MAPK phosphorylation and to alterations in cyclins and CDK pathways and can be significantly inhibited through treatments with inhibitors of MAPK, pathways pathways.49–51 After vascular injury in C57Bl/6 mice, we demonstrate significant formation of neointimal lesions, derived from VSMC (SM-22α positive). In keeping with previous studies, we demonstrate that Cx43 expression as well as MAPK-phosphorylated Cx43 is increased in media and neointimal VSMC in C57Bl/6 mice. However, in Cx43-MK4A mice vascular injury failed to produce significant neointimal formation. Previous studies by others have demonstrated contrasting roles for Cx43 in neointimal formation, with conditional VSMC knockout of Cx43 promoting neointimal formation52 and conversely by others suggesting that reduced Cx43 expression can limit neointimal formation.17 In each of these models, a number of factors could contribute to differences between results including incomplete knockdown of Cx43 in the conditional knockout mouse52 or compensation by other connexins within the Cx43+/− mice as well as differences in model systems, for example, high-fat fed.17 These conflicting results, however, clearly demonstrate the complex nature of Cx43 in regulating VSMC proliferation in response to vascular injury. In comparison to previous models, our Cx43-MK4A mice maintain levels of Cx43 expression within the VSMC of carotids and demonstrate specifically attenuated the neointima formation suggesting that Cx43 is critical in the regulation of neointimal formation. Our data therefore demonstrate that MAPK phosphorylation of Cx43 serines critically regulate VSMC dedifferentiation, proliferation, and neointima formation in vivo.

Our studies demonstrate that Cx43 interacts with cyclin E in a manner that is dependent on MAPK phosphorylation of the Cx43 C-terminus. Previous studies by others have shown that the C-terminus of Cx43 can interact with a number of proteins including tubulin and zonula occludens-1, which are associated with targeting of Cx43 at the plasma membrane.53–55 Further studies have also shown the potential for interactions between the C-terminus of Cx43 and proteins that regulate cell cycle, for example, NOV and SKP2.19,21,56 To define the region on Cx43 where interaction with cyclin E occurs, we generated and purified proteins for the full-length cyclin E and for the Cx43 C-terminus including the null phosphorylation and phospho-mimetic forms. We identified that only Cx43CTMK4D proteins formed interactions with cyclin E. In this in vitro system, we saw no evidence for cyclin E interactions with the native state Cx43CT or the Cx43CTMK4A. By cross-linking proteins in solution from the Cx43CTMK4D samples, we identified higher-molecular-weight forms of both cyclin E and Cx43 at approximately 110 kDa by Western blot. This corresponds to a complex
containing a single cyclin E–GST and a dimer of Cx43. This finding is consistent with previous findings that Cx43 is required to be in a dimeric form to interact with known binding partners.57 In addition to this, we saw no evidence for interaction at higher pH values (pH 7.4, data not shown), where Cx43 is primarily in monomeric forms and is consistent with previously published data.57 Taken together, these data suggest that interactions between MAPK-phosphorylated Cx43 and cyclin E occur within the C-terminus of Cx43 and require the presence of these phosphorylation sites and that these interactions can occur outside of PDGF stimulation in VSMC.

In conclusion, our studies have demonstrated 3 key conclusions: (1) that Cx43 specifically interacts with the cell cycle protein cyclin E in a manner that is dependent on MAPK phosphorylation; (2) that MAPK-phosphorylated Cx43 promotes VSMC proliferation in response to PDGF treatments; and (3) that after vascular injury, MAPK-phosphorylated Cx43 can significantly regulate neointimal formation. Further studies on this interaction may lead to a novel target for therapeutic intervention in the development atherosclerosis and restenosis.

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What Is Known?

- A change in smooth muscle cells from nonproliferative (eg, normal) to proliferative (eg, diseased) states is regulated by mitogen-activated protein kinase pathways (MAPK).
- Alterations in Cx43 protein expression are associated with changes in smooth muscle cells to a proliferative phenotype.

What New Information Does This Article Contribute?

- Cx43 directly interacts with the cell cycle control protein cyclin E.
- Cx43–cyclin E interactions are dependent on the MAPK phosphorylation of Cx43.
- Interactions between Cx43 and cyclin E are critical determinants in smooth muscle cell proliferation and neointimal formation in vivo.

Novelty and Significance

Atherosclerosis has one of the highest mortality rates in the United States, with dysregulated smooth muscle cell proliferation being a hallmark of the disease. Connexin 43 (Cx43) has previously been associated with regulation of cellular proliferation, although the mechanisms have not been described. We identified direct protein interactions between Cx43 and cyclin E, a protein that regulates how cells divide. We specifically demonstrate that phosphorylation of Cx43 protein by MAPK is required for it to bind to cyclin E. Using novel mice in which Cx43 cannot be phosphorylated by MAPK, we further show that this interaction is a critical determinant of smooth muscle cell proliferation and neointimal formation as well as burden of disease. The study has important implications for novel therapeutic strategies.
MAPK Phosphorylation of Connexin 43 Promotes Binding of Cyclin E and Smooth Muscle Cell Proliferation


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Supplemental Material

Online Methods:

Mice: All C57/Bl6 mice (Taconic) used for pluronic applications and carotid ligation injury models were 10-18 week old males and ApoE⁻/⁻ were 15-18 week old males. All mice were kept on a standard 12 hour light/dark cycle and fed a normal chow diet. Food and water was available ad-libatum. Mice containing a heterozygous deletion of Cx43 (Cx43⁺/-, as described)¹ were bred to produce litters containing wild type (Cx43⁺/⁺), heterozygous (Cx43⁺/-) and knockout (Cx43⁻/-) newborns mice for isolation of aortic tissue. Tail snips from each newborn mouse were used for genotyping and loss of Cx43 further confirmed by Western blotting. All animals were used according to the University of Virginia Animal Care and Use Committee guidelines as previously described.²

The Cx43 MAPK knock-in mice (Cx43-MK4A) were made using a v6.5 ES cell line derived from C57Bl/6J and 129S6SvEV F1 hybrid mice. The knock-in vector contained a 5' homology arm, the non-coding Exon 1, a neo/PGK cassette flanked by loxP sites followed by Exon2 that contains the whole Cx43 coding sequence including the mutations (S255A, S262A, S279A and S282A) and a 3' homology arm. After electroporation, ES cells were screened with long range PCR using primers amplifying from the homology arms to the floxed neomycin cassette, and homologous recombination was confirmed by Southern blotting to ensure intact homologous recombination into the Cx43 locus (Online Figure I). The mice were interbred with B6.C-Tg (CMV-cre) 1Cgn/J mice (Jackson) to remove the neomycin selection cassette and then backcrossed to C57Bl/6J mice 6 times to omit the Cre gene and approach strain homogenicity. All mice used for experimentation were 10-18 week old males.

Vascular smooth muscle cell (VSMC) isolation: Newborn mice were euthanized by decapitation and descending aortas removed, placed into a wash solution (HBSS,1% BSA, 1% Glutamine, 1% sodium pyruvate, L-ascorbic acid w/v 10%w/v, pyruvate 20% w/v, Fungizone 0.1%), and dissected horizontally and longitudinally. Explants, were placed in 75 mm flasks containing 2 mL of Amniomax C-100 media plus supplement (Gibco), and incubated at 37°C, 5% CO₂ for 2 weeks with media changed every 3 days. After VSMC outgrowth, explants were removed and media replaced with fresh supplemented Amniomax media for 1 week (Online Figure II). Cells were trypsinized, re-plated in the same 75mm flask (Passage 1, P1) and grown to near confluence. Media was then exchanged for DMEM F12 containing 20% FBS (Defined, Hyclone) with 1% pen/strep, 1% glutamine for 48 hour, VSMC trypsinized and re-plated in 225 mm flasks (P2) and grown to near confluence. Finally media was exchanged for growth media (10% FBS in DMEM F12 with 1% pen/strep, 1% glutamine) for 48 hours before cells being trypsinized and re-plated into 225 mm flasks (P3). Cells were used between P4 and P8 for all experiments.

Carotid surgeries: Pluronic application surgeries were performed as described.² Briefly, mice were anesthetized by intraperitoneal injection of ketamine-xylazine, the left common carotid artery exposed, and PDGF (200 ng) mixed in pluronic gel (F-127) was applied to the outside of the carotid wall. Forty-eight hours after the surgery, mice were euthanized with an intraperitoneal injection of 60-90 mg/kg pentobarbital, mice were perfused with 4% PFA and both treated (left common) and control (right common) carotids removed and stored in 4% PFA overnight for whole mount analysis or immuno-sectioning. For ultrastructure transmission electron microscopy (TEM) and immune-TEM (i-TEM) vessels were fixed in 4% PFA containing gluteraldehyde (2.5% or 0.5% respectively). For in vivo cell proliferation studies, mice were pre-
injected with 5-ethynyl-2'-deoxyuridine (EDU) prior to surgeries and carotids removed post-treatment for whole mount analysis of EDU incorporation into VSMC (n=4, as described).²

Carotid ligation (injury) surgeries were performed as previously described.³ Briefly, mice were anesthetized by intraperitoneal injection of ketamine-xylazine, the left common carotid artery exposed and carefully freed from surrounding tissue. A 6/0 silk suture was run under the carotid artery distal to the bifurcation site and two consecutive, flat square knots were tied tight enough to restrict blood flow. Control mice were surgical controls, where carotid artery was exposed and freed of surrounding tissue as described above, but no suture was applied. At 2 weeks after surgery, mice were euthanized with an intraperitoneal injection of 60-90 mg/kg pentobarbital, mice were then perfused with a krebs buffered solution containing heparin followed by 4% PFA in kreb solution. Carotids were then removed and stored in 4% PFA overnight for whole mount analysis or immuno-sectioning. We characterized neointima formation in C57Bl/6 mice to occur at between 1-3mm distal of the ligation site as has been previously described.³ For analysis of neointima formation in vessels, areas corresponding to the adventitia (immediately surrounding the media), the media (containing VSMC), neointima (containing VSMC) and lumen were measured on H&E stained cross sections of carotid vessels were measured using MetaMorph image analysis software (n=7, Online Figure III).

In-vitro cell proliferation: VSMC were plated at 5x10⁴ cells in 6 well plates for 24 hours in growth media and cell cycle stall induced by switching to a low serum media (0.5% FBS) for 48 hours followed by transfection with plasmids (5 µg DNA each, Cx43² VSMC only,) using Lipofectamine 2000 for 18 hours. Cells were then incubated with EDU (5 µg/mL) with or without the addition of PDGF (10 ng/mL) for 24 hours in low serum media. In studies of reduced gap junction communication, carbenoxolone (25 mM) was applied to cells for 30 mins prior to the addition of PDGF and EDU for 24 hours. Cells were trypsinized, pelleted, re-suspended in 0.5 mL PBS then fixed using 4% PFA and stored at 4°C. Cell proliferation was measured by flow cytometry of Alexa 488 labeled EDU as previously described.²

Plasmids: A DNA construct for full length rat Cx43 (Cx43, a.a.1-382,⁴ Online Figure IV) in vector pcDNA3.1 was grown in DH5α cells (Invitrogen). In experiments, control vector was empty pcDNA3.1. The Cx43 C-terminus (Cx43²⁵, rat, a.a. 236-382, Online Figure IV) DNA construct sub-cloned in frame with the glutathione S-transferase (GST) tag in pGEX-6P-2 plasmids was kindly provided by Dr P. Sorgen (as described ⁵) and was grown in BL21-codon plus cells (Stratagene/ Agilent technologies< Santa Clara, CA). Full length human cyclin E (cyclin E1, transcript variant 2, NM 057182.1) inserted in the pGEX-2T plasmid, kindly provided by Dr B. Lüscher,⁶ was transformed in BL21-codon plus cells. All DNA sequencing was performed by the UVA sequencing core facility.

Site directed mutagenesis: Primers for site directed mutagenesis of Cx43/ Cx43²⁵ were designed using QuikChange primer design program and mutagenesis performed using the QuikChange kit (Agilent, Stratagene, Santa Clara, CA). Primers were designed against MAPK serines (Cx43: S255, S262, S279, S282 or corresponding Cx43²⁵: S20, S27, S44, S47), to alanine (non-phosphorylated, Cx43MK⁴⁴⁴/Cx43CTMK⁴⁴⁴) or to aspartate (phospho-mimetic, Cx43MK⁴⁴⁴/Cx43CTMK⁴⁴⁴) See Online Figure IV. To produce Cx43²⁵ constructs used for transfection of Cx43²⁵ cells a stop codon was inserted immediately 5’ of a.a. 382 of the Cx43²⁵, Cx43³⁸²⁵ and Cx43³⁸²⁵ constructs by site directed mutagenesis. Constructs were then excised using BamH1 and Xho1 from the pGEX-6P-2 vector and ligated to the pcDNA3.1 vector. Following mutagenesis, plasmids were sequenced using primers designed against the T7 promoter in the pcDNA3.1 vector and against Cx43 (for full length Cx43) or against the pGEX expression vector or the T7 promoter for Cx43²⁵ plasmids.
Bacterial protein expression and purification: Connexin 43<sup>CT</sup> proteins were purified as described<sup>5</sup> with the following alterations to the protocol. Plasmids were transformed into BL21 competent bacterial cells. Bacterial clones grown in 2 L of LB broth were induced at 0.5 OD<sub>600</sub> with 1 mM isopropanol-1-thio-B-D-galactopyranoside (IPTG) for 3 hours. Bacteria were pelleted and re-suspended (1 g/ 5 mL) in protein buffer: PBS (pH 7.4); NaCl (190 mM); dithiothreitol (DTT, 1mM); Pefabloc (0.1 mM) and complete protease inhibitor cocktail (1:100, Sigma) then stored at -80 °C. Cells were thawed and membranes disrupted by microfluidics then lysates cleared by centrifugation (100,000 x g, 1 hour, 4°C). Supernatants were incubated with 4 mL of glutathione-sepharose beads (Glutathione-sepharose 4 fast flow, GE Lifesciences) for 3 hours, flow rate 0.5 mL/min at 4°C. Protein bound beads were washed with 40 column volumes of protein buffer, followed by 20 column volumes of protein buffer without Pefabloc or protease inhibitors and GST cleavage performed by incubation with PreScission (80U) at 4°C overnight with rocking. Eluted Cx43<sup>CT</sup> proteins were further purified by size exclusion (320 mL Superdex 75 size exclusion column, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) to greater than 90% purity. Eluted Cx43<sup>CT</sup> samples were collected and concentrated using Amicon centriplus 10 filters.

Cyclin E was prepared as described<sup>7</sup> with the following modifications. Cyclin E transformed BL21 bacterial cells were grown overnight then media inoculated to 0.1 OD. Bacteria were grown in 4 L to 1.0 OD at 37 °C, chilled to 16 °C on ice, grown at 16 °C for 1 hour then protein expression induced with IPTG (0.1 mM) for 24 hours at 16 °C. Bacteria were then pelleted and re-suspended in protein buffer and protein purification was performed as described for Cx43<sup>CT</sup> proteins except cyclin E-GST was eluted from the beads (without cleavage) in a solution of PBS plus L-glutathione (1 mM) prior to size exclusion purification (as above).

Analytical size exclusion chromatography (ANSEC): Prior to analysis, sample buffers were altered to pH 6.5, KCl (40 mM) with no DTT using PD10 de-salting columns (GE Lifesciences) followed by concentration of proteins to approximately 15-20 mg/mL. Proteins analyzed by ANSEC were at a final concentration of 350 µM for Cx43<sup>CT</sup> and 75 µM for cyclin E-GST with a molar ratio of 4.7:1 (43<sup>CT</sup>: cyclin E-GST) for combined samples. Samples were run on a Bio-Silect SEC 250-5 column with a bed volume of 14 mL (300x7.8 mm, Biorad; Hercules, CA) either as single proteins or as Cx43<sup>CT</sup> + cyclin E-GST co-incubated for 5 minutes prior to loading on ANSEC column. Eluted fractions from the column were analyzed by Western Blot for the presence of Cx43 and cyclin E. Molecular weight standards of blue dextran (~2,000 kDa; void volume), alcohol dehydrogenase (150 kDa) and albumin (66 kDa) were run using the above conditions and eluted in fractions 11, 16 and 17, respectively.

Cell culture, total protein isolations: Following treatments, all cells were harvested in cold lysis buffer 1: PBS (pH 7.4) containing: NaCl (125 mM); EDTA (5 mM); sodium deoxycholate (1%); triton X-100 (0.5%); sodium orthovanadate (500 µM); AEBSF (10 µM); protease inhibitor cocktail (1:100, Sigma) and phosphatase inhibitor cocktail 2 + 3 (1:100, Sigma). All isolations were performed at 4 °C, samples were dounced 30 times on ice, incubated with rotation for 30 mins at 4 °C and centrifuged at 14,000g for 5 minutes. Cleared lysates were used for Western blot and co-immunoprecipitation analysis. For experiments investigating Retinoblastoma (Rb) and its phosphorylation by Western blot, total protein lysates were sonicated immediately following harvest in lysis buffer 1 and used without centrifugation. Proteins samples were quantified by bradford assay prior to loading and equal loading confirmed using GAPDH or α-tubulin.

Cell culture, membrane preparations: Membrane fractions were harvested as previously described<sup>2</sup> Briefly, following treatments all cells were harvested in lysis buffer 2: PBS (pH 7.4)
containing: NaCl (125 mM); sodium orthovanadate (500 µM); AEBSF (10 µM); protease inhibitor cocktail (1:100, Sigma) and phosphatase inhibitor cocktail 2 + 3 (1:100, Sigma). All isolations were performed at 4 °C, samples were dounced 30 times on ice, incubated with rotation for 30 mins at 4 °C and centrifuged at 14,000g for 5 minutes. Cleared lysates were then centrifuged at 100,000g at 4 °C for 1 hour and pellets (membrane fractions) resuspended in lysis buffer 1. Harvested membrane fractions were dounced 30 times on ice, incubated with rotation for 30 mins at 4 °C and centrifuged at 14,000g for 5 minutes. Cleared lysates were used for Western blot and co-immunoprecipitation analysis. Proteins were quantified by bradforfs assay prior to loading and equal loading confirmed on Western Blot using GAPDH as previously described for membrane proteins. Membrane sample purity was determined by through lack of detection of α-tubulin by Western blot.

Co-immunoprecipitation: Vascular smooth muscle cells (Cx43+/−) were switched to low serum media for 72 hours prior to treatment with PDGF (10 ng/mL) for 24 hours. The VSMC isolated from Cx43+/− in growth medium were transfected by Nucleofector (Lonza) using 10 µg of each plasmids for 18 hours followed by incubation with PDGF for 24 hours. Following treatments all cells were harvested as described above for total protein lysates or membrane proteins. Dynabeads coated with antibodies for either Cx43, cyclin E or CDK 2 were incubated with lysates for 3 hours at 4 °C followed by 4 washes in lysis buffer 1 prior to elution of bound proteins from the beads using 5x lamelli buffer for Western Blot analysis (as described).

Scrape load dye transfer (SLDT): Cells stalled were transfected (as above, using Lipofectamine) then treated with PDGF (10 ng/mL), carbenoxolone (25 µM) plus PDGF, or no treatment (control). Twenty four hours after treatment, lucifer yellow (0.5 mg/mL in PBS) was applied to cells then a scrape was created using a 23G needle, with 3 scrapes per well. After 10 minute incubation at 37°C, cells were washed in PBS and fixed for 5 mins in 4% PFA (as described). Images of dye transfer were captured on an Olympus FVX with a 20x 0.6 NA water immersion lens. Distance of dye transfer was quantified from the wound edge using MetaMorph imaging software.

Cell Treatments: For inhibition of Cx43:cyclin interactions, cell cycle stalled Cx43+/− VSMC were pre-treated with Erk inhibitors, U0126 (10 µM, VWR, kindly provided Dr Norbert Leitinger) or Roscovitine (15 µM, Selleck) or with DMSO (1 µl/mL, control) prior to treatment with PDGF (10 µg/mL).

Antibodies: Immunocytochemistry, i-TEM and Western blot analysis of samples were performed using antibodies against: Cx43 (polyclonal, Sigma); MAPK phosphorylated-Cx43 (Cx43-P, polyclonal); cyclin E (monoclonal and polyclonal Abcam); cyclin D1 (monoclonal Abcam); p21 (monoclonal, Santa Cruz); p27 (monoclonal, Santa Cruz); CDK2 (monoclonal, BD biosciences); eNOS (polyclonal, BD biosciences); prolly-4-hydroxyase (polyclonal, Abcam); SM α-actin (polyclonal, sigma); SM-22α (polyclonal, Abcam); total Retinoblastoma (Rb clone 4H1, polyclonal Cell Signalling); phosphorylated Rb (polyclonal Cell Signalling); phosphorylated Rb (monoclonal, Cell Signalling). Antibodies were visualized with donkey anti-rabbit or anti-mouse Alexa 594/ Alexa 488 for immunocytochemistry or using 680/800 nm conjugated secondary antibodies (LI-COR) for Western blotting.

Immunocytochemistry: All immunofluorescence was performed as described. In all images Cx43 and cyclin E were detected using secondary antibodies coupled to Alexa 594 and Alexa 488 respectively with nuclei identified through 4',6-diamidino-2-phenylindole (DAPI) staining. For quantification of pixel intensities in carotids, regions corresponding to VSMC layers were placed between layers of elastic lamina using MetaMorph imaging software (as described). In
each image, at least three areas of VSMC were recorded; at least three images were used per mouse per treatment (n = 4).

**Transmission electron microscopy:** Carotid samples (isolated as above) were analyzed by TEM or i-TEM as previously described. Analysis of protein interactions on i-TEM was performed using differing sized immuno-gold beads to detect: Cx43/ Cx43-P (25 nm), CDK2 (15 nm) and cyclin E (10 nm). To determine the distance relationship between the proteins, the distance between the edges of each bead type and its closest surrounding bead was measured using Metamorph imaging software. An n=20 interactions was measured for each treatment with representative images shown.

**Statistical analysis:** 1-way or 2-way ANOVA followed by Bonferroni’s post-test were used for comparisons between 3 treatment groups and student t-test used for comparisons of 2 treatment groups. A $P$ value of $<0.05$ was significant, in all images * is $P < 0.05$, ** is $P<0.01$ and *** is $P<0.001$. 
Online Figure IV

A

Cx43: a.a. 1-382
Cx43<sub>CT</sub>: a.a. 236-382

B

Mutations:

Cx43
Cx43<sub>CT</sub>

Cx43<sub>MK4D</sub>

Cx43<sub>CTMK4D</sub>

Cx43<sub>MK4A</sub>

Cx43<sub>CTMK4A</sub>
Online Figure V

- **Input**
  - 0.5% 10% Cx43
  - cyclin E
  - GAPDH

- **IP**
  - 0.5% 10% IgG
    - IP: cycE
    - WB: Cx43
    - IP: cycD1
    - WB: Cx43
    - IP: Cx43
    - WB: cycE
    - IP: Cx43
    - WB: Cx43
    - IP: p21
    - WB: Cx43
    - IP: p27
    - WB: Cx43
    - IP: Cx43
    - WB: p27

- 25kDa 37kDa 50kDa
### Online Table I

#### Cx43 Input

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#### Cx43 IP

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#### Ratio (IP:Input)

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Online Figure Legends

Supplementary Figure I: Generation of Cx43-MK4A mice: In A, the scheme for the knock-in vector highlights site of insertion of Cx43 knock in (KI) coding sequence including the mutations (S255A, S262A, S279A and S282A). PCR analysis of DNA samples taken from wild type (wt), heterozygous and Cx43-MK4A demonstrates the slower migrating band that is seen for Cx43-MK4A construct (B).

Supplementary Figure II: Isolation of aortic VSMC. Newborn mice (around 2 hours old) taken from breeding pairs of Cx43+/− mice were euthanized by decapitation and the descending aortas removed (A). Explants were plated in supplemented Amniomax media and VSMC sprouting observed over a 2 week period before removal of explants and re-plating of VSMC (Passage 1, B). Cells were confirmed to be VSMC by immunofluorescence based on the presence of SM α-actin (Red, C) with nuclei labeled using DAPI (Blue, C). In B the scale bars are: top left is 200 μm, top right and lower left are 50 μm and in C the scale bar is 10 μm. Cells isolated from newborn mouse aortas of C57Bl/6 mice were confirmed to be VSMC through the absence of the endothelial cell marker eNOS as shown in endothelial cells (EC, HUVEC cells), the absence of fibroblast marker prolly-4-hydroxylase as shown in primary human fibroblasts (Fibroblast) and by the presence of smooth muscle markers SM α-actin and SM-22α (D). These studies identified that cells isolated from the aortas of newborn mice were primarily VSMC. In D, black arrowheads identify the expected molecular weight for each protein, colored arrowheads correspond to molecular weights: green is 25 kDa, blue is 37 kDa, red is 50 kDa, Yellow is 100 kDa.

Supplemental Figure III: Vessel wall measurements in carotid ligation mice. Areas corresponding to the adventitia (A), media (M), neointima (N) and lumen (L) were measured on H&E sections from control and injury mice for C57Bl/6 and Cx43-MK4A mice with MetaMorph imaging software (A). In A the scale bar is 100 μm. Areas were converted from pixels to mm² and statistical analysis performed comparing controls versus injury in C57Bl/6 and Cx43-MK4A mice, n=7 for each condition (B). In C57Bl/6 mice following injury, we observed increases in adventitial and media thicknesses as well as the development of neointima and consequently reductions in lumen diameter. In Cx43-MK4A mice, we did not observe thickening of the adventitial layer or significant formation of neointima (B). However, alterations within the media layer were evident with an increased media thickness, which was significantly less than in C57Bl/6 mice (B). In graphs, *** indicates P<0.05 and **** indicates P<0.001 (B, n=7).

Supplemental Figure IV: Plasmid generation and mutagenesis. Plasmids for full length Cx43 (Cx43, a.a. 1-382, pCDNA 3.1) or Cx43 C-terminus (Cx43_CT, a.a. 236-382, pCDNA3.1 or p-GEX6P2) (A) were sequenced then mutagenesis performed to produce the phospho-mimetic form (Aspartate, D) or null phosphorylation forms (Alanine, A) at the MAPK serines representative of S255, S262, S279 and S282 in the C-terminus (B). Amino acid regions highlighted in green show the known α-helical regions within the Cx43 C-terminus.

Supplementary Figure V: Cx43 interacts with cyclin E in aortic smooth muscle cells. Mouse aortic VSMC (Cx43+/−) were stalled in 0.5% serum media for 72 hours followed by 24 hours in either 0.5% or 10% serum as indicated. Cells were lysed in lysis buffer 1 and cleared lysates incubated with Dynabeads coated with Cx43, cyclin E, cyclin D1, p21waft/cip1 or p27kip1 antibodies as labeled. Proteins eluted from beads were analyzed by Western blot using antibodies directed against Cx43, cyclin D1, cyclin E, p21waft/cip1, p27kip1. Representative Western Blots (input) demonstrate that in cells treated with 10% serum, expression levels of Cx43, cyclin E and CDK2 are increased, whereas expression of the cell cycle inhibitors p21waft/cip1, p27kip1 are decreased. Co-Immunoprecipitation (IP) studies demonstrate that of the proteins tested only Cx43 and cyclin E form in complex in proliferating cells (10% serum). Black
arrowheads identify the expected molecular weight for each protein, colored arrowheads correspond to molecular weights: green is 25kDa, blue is 37 kDa, red is 50kDa.

**Supplemental Figure VI: PDGF promotes formation of complexes between Cx43, cyclin E and CDK2 in vitro.** Mouse aortic VSMC (Cx43+/+) were stalled in 0.5% serum media for 72 hours followed by 24 hours in either 0.5% or 10 ng/mL PDGF in 0.5% serum as indicated. Cells were lysed in lysis buffer 1 and cleared lysates were incubated with dynabeads coated with Cx43, cyclin E or CDK2 antibodies as labeled. Proteins eluted from beads were analyzed by Western blot using antibodies directed against Cx43, cyclin E or CDK2. Representative Western Blots (input) demonstrate that in cells treated with PDGF, expression levels of Cx43, cyclin E and CDK2 are increased. Co-Immunoprecipitation (IP) studies demonstrate that Cx43, cyclin E and CDK2 form in complex in PDGF treated cells. Black arrowheads identify the expected molecular weight for each protein, colored arrowheads correspond to molecular weights: green is 25kDa, blue is 37 kDa, red is 50kDa.

**Supplemental Figure VII: Effects of transfection on gap junction communication.**
Confluent monolayers of Cx43+/+ or Cx43−/− cells in low serum were transfected with Cx43 plasmids as described in supplemental materials and lucifer yellow dye transfer was assessed by SLDT in control (untreated) or PDGF treated cells (A). In both untreated Cx43+/+ and Cx43−/− cells, dye transfer from the edge of the wound to surrounding cells was identified. Transfection of Cx43−/− cells with plasmids containing full length Cx43 did not produce significant alterations in dye transfer in untreated or PDGF treated conditions (A-B). Reductions in dye transfer were observed following treatments of Cx43−/− cells with the gap junctional inhibitor CBX (25 μM) in both untreated and PDGF treated cells (A). In B representative images show dye transfer in vector transfected, CBX treated Cx43−/− cells and in untreated and PDGF treated Cx43 transfected Cx43−/− cells. In B the dashed line represents the edge of the scrape, scale bar is 40 μm. Cx43+/+ cells pre-treated CBX (25 μM) did not demonstrate significantly reduce VSMC proliferation following treatment with PDGF as compared to PDGF treated (C). In graphs, “*” indicates P<0.05 and “***” indicates P<0.001 (A, n=3 and C, n=6)

**Supplemental Figure VIII: Expression of free Cx43CT proteins in Cx43−/− VSMC.** Mouse aortic VSMC (Cx43−/−) in low serum were transfected with Cx43CT+ plasmids as described in supplemental materials. Following PDGF treatments cells were lysed in lysis buffer 1 and cleared lysates were incubated with dynabeads coated with cyclin E antibodies. Proteins eluted from beads were analyzed by Western blot using antibodies directed against Cx43. Representative Western Blots (input) demonstrate that free Cx43CT+, Cx43CTMK4A+, Cx43CTMK4D+ proteins can be expressed in the Cx43−/− cells. Co-Immunoprecipitation (IP) studies demonstrate that free Cx43CT+ proteins do not co-precipitate with cyclin E in PDGF treated Cx43−/− cells (A). VSMC proliferation was increased in Cx43−/− cells transfected to express full length Cx43 but not free Cx43CT+, Cx43CTMK4A+, Cx43CTMK4D+ proteins in response to PDGF treatments (B). Comparisons of proteins isolated form transfected Cx43−/− cells demonstrate the both full length and free Cx43CT proteins can be identified in total protein lysates but only full length proteins can be identified in the cell membranes (C). In A and C, black arrowheads identify the expected molecular weight for each protein, colored arrowheads correspond to molecular weights: green is 25kDa, blue is 37 kDa, red is 50kDa. In graphs “****” indicates P<0.001 (B, n=4)

**Supplemental Figure IX: Effects of PDGF treatments on cyclin E and CDK2 in Cx43−/− cells.** Mouse aortic VSMC (Cx43−/−) grown in low serum for 72 hours were treated with PDGF and harvested at specific timepoints over the course of 24 hours as described in supplemental materials and methods. Cells were lysed in lysis buffer 1 and cleared protein lysates were analyzed by Western Blot for cyclin E an CDK2 expression. PDGF treatments did not produce notable increases in cyclin E expression but CDK2 proteins were found to be increased by 24 hours.
Supplemental Figure X: Cx43 expression in Cx43-MK4A carotid VSMC. Carotid sections were analyzed by immunofluorescence for protein expression using antibodies directed against Cx43. In each image red represents Cx43, green represents internal elastic lamina, blue indicates nuclei (DAPI) and **" represents the luminal side of the vessels. Scale bar is 20 μm. Immunofluorescence quantification of Cx43 (B) and SM22-α (C) from carotid VSMC layers. Treatment of carotids with PDGF increases Cx43 expression in VSMC from C57Bl/6 but not Cx43-MK4A mice (n=4).

Supplemental Figure XI: Cx43 interactions in Cx43-MK4A carotid VSMC. Carotid sections from Cx43-MK4A mice were analyzed by immunofluorescence for protein expression and co-localization using antibodies directed against Cx43, cyclin E or CDK2. In each image red represents Cx43, green represents either cyclin E or CDK2 (as labeled), blue indicates nuclei (DAPI) and **" represents the luminal side of the vessels, scale bar is 20 μm. No co-localization (orange) was detected in either control or treated vessels from Cx43-MK4A mice.

Supplemental Figure XII: Schematic of Cx43 phosphorylation and control of VSMC proliferation. VSMC proliferation in atherogenesis is associated to increases in PDGF and alterations in the expression of Cx43 (A). PDGF which acts via its receptor activates an ERK signaling pathway that induces phosphorylation of Cx43 at the MAPK serines (B). Following phosphorylation of Cx43 MAPK serines (C1), both cyclin E and CDK2 interact with the C-terminus of Cx43 (C2). Following interactions, the protein complex becomes internalized (C3). The interaction of Cx43 and cyclin E is critical in the regulation of VSMC proliferation (C4).

Online Table I: Quantification of Cx43 expression in transfected Cx43−/− cells. Western blot band intensities for Cx43 protein in transfected (top) and transfected co-immunoprecipitation (middle) samples were used to calculate a ratio (IP: Input, bottom) for each sample. Samples were quantified using Licor Odyssey imaging software and analysis performed by Origin Pro software (n=3).
Supplemental Method References


