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Circ Res. 2008;102:653-660; originally published online January 31, 2008;
doi: 10.1161/CIRCRESAHA.107.170472
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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http://circres.ahajournals.org/content/102/6/653

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Targeting Connexin 43 Prevents Platelet-Derived Growth Factor-BB–Induced Phenotypic Change in Porcine Coronary Artery Smooth Muscle Cells

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Abstract—We previously reported that reducing the expression of the gap junction protein connexin (Cx)43 in mice restricts intimal thickening formation after acute vascular injury by limiting the inflammatory response and the proliferation and migration of smooth muscle cells (SMCs) toward the damaged site. SMC populations isolated from porcine coronary artery exhibit distinct phenotypes: spindle-shaped (S) and rhomboid (R). S-SMCs are predominant in the normal media, whereas R-SMCs are recovered in higher proportion from stent-induced intimal thickening, suggesting that they participate in the restenotic process. Here, we further investigate the relationship between connexin expression and SMC phenotypes using porcine coronary artery SMCs. Cx40 was highly expressed in normal media of porcine coronary artery in vivo, whereas Cx43 was barely detectable. In contrast, Cx40 was downregulated and Cx43 was markedly upregulated in stent-induced intimal thickening. In vitro, S-SMCs expressed Cx40 and Cx43. In R-SMCs, Cx43 expression was increased and Cx40 was absent. We confirmed that S-SMCs treated with platelet-derived growth factor-BB acquire an R phenotype. This was accompanied by an upregulation of Cx43 and a loss of Cx40. Importantly, platelet-derived growth factor-BB–induced S-to-R phenotypic change was prevented by a reduction of Cx43 expression with antisense, ie, S-SMCs retained their typical elongated appearance and the expression of α-smooth muscle actin, a well-known SMC differentiation marker, whereas the expression of S100A4, a typical marker of R-SMCs, was prevented. In conclusion, limiting Cx43 expression in S-SMCs prevents platelet-derived growth factor-BB–induced S-to-R modulation. This suggests that Cx43 may be an additional target for local delivery strategies aimed at reducing restenosis. (Circ Res. 2008;102:653-660.)

Key Words: gap junction ■ connexin ■ smooth muscle cell ■ atherosclerosis ■ restenosis

During atherosclerotic lesion formation or restenosis after angioplasty or stent application, smooth muscle cells (SMCs) migrate from the media to the intima, where they proliferate and undergo phenotypic changes. The mechanisms that regulate this process and the origin of the intimal SMCs have been intensely investigated and the subject of much debate in recent years. The original hypothesis described that the combined action of growth factors and cytokines produced by a dysfunctional endothelium and inflammatory cells induces the migration of medial SMCs as well as their proliferation. This concept implies that all SMCs of the media can undergo the phenotypic modulation from a contractile, ie, differentiated to a synthetic, ie, dedifferentiated phenotype. Based on the work of Benditt and Benditt, later confirmed by Murry et al, who reported that human atherosclerotic plaques have the features of a monoclonal or an oligoclonal lesion, the present paradigm is that a predisposed medial SMC subpopulation is responsible for intimal thickening (IT) formation.

The concept of SMC phenotypic heterogeneity has been validated in several species including human and porcine coronary artery (CA) (reviewed elsewhere). Indeed, we have recently isolated 2 distinct SMC populations from the normal porcine CA media: spindle-shaped (S), with the classic “hills-and-valleys” growth pattern, and rhomboid (R), growing as a monolayer. R-SMCs show increased proliferative, migratory, and proteolytic activities and are poorly differentiated compared with S-SMCs. Moreover, R-SMCs are obtained in higher proportions when SMCs are isolated from stent-induced IT compared with the normal media, suggesting that they are involved in arterial repair and restenosis. Recently, we have identified S100A4, a protein that belongs to a large family of low-molecular-weight Ca2+-binding proteins, as a marker of R-SMCs and of activated SMCs in atherosclerosis and restenosis.

Vascular SMCs are linked by gap junctions, clusters of transmembrane channels that act as conduits for the direct...
intercellular exchange of ions and small signaling molecules between neighboring cells (reviewed elsewhere). The individual components of these channels, connexins (Cx), form a multigene family of conserved proteins, of which more than 20 members have been identified in mammalian cells. The major Cx expressed by medial SMCs of large and medium-sized arteries is Cx43. In addition, Cx40 is observed in medial SMCs of small elastic, muscular, and resistance arteries. Other Cxs, ie, Cx45, Cx37, and Cx31.9, seem to be confined to specific locations of the vascular tree. Gap junctional intercellular communication (GJIC) between SMCs has been implicated in the maintenance of circulatory homeostasis, coordination of vasomotor responses (reviewed elsewhere), and coordination of SMC differentiation during vascular development. Increasing evidence demonstrates that Cx43-mediated communication between SMCs may also play a role in the vascular response to acute injury or chronic inflammation. Thus, increased intimal expression of Cx43 has been observed during the growing phase of human and mouse atherosclerotic lesions, and reducing Cx43 restricts atherosclerotic plaque development in mice. Similarly, balloon distension, as well as wire injury in animal models, results in increased Cx43 expression in the intima, and reducing Cx43 expression changes the course of the restenotic process in mice. It is of interest to study the effects of Cx43 modulation in 2 distinct medial SMC populations, of which 1 displays an IT-prone phenotype.

The present study set out to examine the relationship between Cx expression and SMC migration and phenotype using S- and R-SMC populations. Inhibition of GJIC in these cells was achieved using the pharmacological gap junction blocker 18-α glycyrrhetinic acid (α-GA), Cx43-specific blocking peptides, or Cx43 antisense. R-SMCs displayed higher Cx43 expression levels and more cell-to-cell coupling than S-SMCs. Furthermore, R-SMCs did not express Cx40, an additional gap junction protein found in S-SMCs. Interestingly, PDGF-BB–induced S-to-R modulation and migration of S-SMCs were prevented by Cx43 inhibitory strategies. Our findings suggest that Cx43 may be an attractive target for local delivery strategies aimed at reducing restenosis.

Materials and Methods

Arterial Specimens

Animal procedures were performed according to the Swiss Federal Veterinary Guidelines and approved by the Ethics Committee of the Geneva Medical School. Three-month-old domestic crossbred female pigs (Sus scrofa) were used. IT was induced by direct self-expanding stent implantation (Wallstent, Schneider, Bulach, Switzerland) in the left anterior descending and circumflex CA. Nonstenosed left anterior descending, circumflex, and right coronary vessels served as controls. Injured vessels were collected 10 and 30 days after stent implantation, and tissue specimens were snap-frozen in OCT resin (Tissue-Tek).

Cell Culture

Cxs of 8-month-old pigs were obtained from a local slaughterhouse. SMCs with different phenotypes were isolated from the media using enzymatic digestion (S-SMCs) or tissue explantation (R-SMCs), as previously described. SMC populations (N=6 for each phenotype) were maintained in DMEM (GIBCO-BRL) containing 10% FCS (Amimed). Cultured S-SMCs were treated for 7 days with human recombinant PDGF-BB (10 ng/mL, Roche) in DMEM supplemented with 10% FCS to induce phenotypic changes.

Immunofluorescence Staining

Serial cryosections (5 μm) were obtained from control or injured CAs and immunostained with antibodies recognizing Cx43 (Cx43B12-A, ADI, San Antonio, Tex), Cx40 (Cx40-A, ADI), or Cx37 (Cx37A11-A, ADI), as previously described. Slides were mounted with Vectashield mounting medium (Vector laboratories, Burlingame, Calif) and examined with an TMD300 microscope (Nikon AG, Küsnacht, Switzerland) equipped with a high-sensitivity charged-couple device Visi kam camera (Visitron Systems GmbH, Puchheim, Germany) connected to a personal computer. Images were captured using the software MetaFluor 4.01 (Universal Imaging Corp, Downingtown, Pa) and processed using Adobe Photoshop. S- and R-SMCs were plated on glass coverslips and immediately used for immunostaining using the abovementioned connexin-specific antibodies and protocols. Negative controls included omission of first antibodies or preincubation of first antibodies with immunogenic peptides. A mouse endothelial cell line (bEnd.3) was used as positive control for the immunostaining.

Migration Assays

Migration assays were performed using 12-transwell plates (polycarbonate filter, 8-μm pore size; Vitrasis) as previously described. Briefly, migration of 5×10⁴ cells per well was assayed for 16 hours at 37°C using 5 or 10 ng/mL human recombinant PDGF-BB as a chemoattractant. In some experiments, SMCs were preincubated with Cx43 sense or antisense (100 μmol/L) phosphorothioated oligonucleotides for 24 hours or with Cx43-specific blocking peptide (190 μmol/L) for 20 minutes before migration assay. The optimal concentration of Cx43 antisense was determined by immunofluorescence staining after incubation of subconfluent R-SMC cultures for 24 hours with 1, 5, 10, 20, 50, or 100 μmol/L of the oligonucleotides. Cell migration was quantified in triplicate for each experiment.

Western Blotting

Western blotting of protein extracted from primary SMCs was performed as described using antibodies against Cx43 (BD Transduction), Cx40 (Chemicon), α-smooth muscle actin (SMA) (clone 1A43), and S100A4 (clone 4B48). A mouse monoclonal IgG1 (clone AC-1536) specific for Cxs. Extracted from bEnd.3 cells were included as positive control for Cxs.

Dye Coupling

Dye transfer assays with 4% Lucifer yellow on subconfluent cultures of S- and R-SMCs were performed as previously described.

Statistical Analysis

Results are presented as means±SEM. Unpaired t test was used to compare differences between 2 groups, and ANOVA was used for comparison of multiple groups. Data were considered statistically significant at P<0.05.

Results

Cx Expression After Stent Implantation in Porcine CAs

Histological appearance of control CAs and CA segments with stent-induced IT at 10 and 30 days stained with hematoxylin/eosin are shown in Figure 1 (left images). Immunofluorescence staining of control and injured vessels 10 and 30 days after stent implantation showed that Cx43 was...
modestly expressed in normal media, with a scattered pattern of expression (Figure 1, middle images). Ten days after stent implantation, Cx43 expression was strongly upregulated in the IT and remained scattered in the underlying media. Thirty days after stent implantation, Cx43 expression remained appreciable with a more scattered pattern. In contrast to Cx43, Cx40 expression was prominent in control CAs but was absent in the IT 10 days after stent implantation (Figure 1, right images). Thirty days after stent implantation, Cx40 expression was weak and scattered in the IT. Cx37 was not detected in any condition (data not shown). These data demonstrate that Cx expression is modulated during the process of restenosis in CAs.

**Cx Expression in S- and R-SMCs**

As previously described, the S-SMCs exhibited a classic “hills-and-valleys” growth pattern at confluence (Figure 2A, top left), whereas R-SMCs had a polygonal and flat appearance and grew to a monolayer at confluence (Figure 2A, top right). Immunofluorescence staining performed on SMC populations showed that Cx43 and Cx40 were expressed in S-SMCs (Figure 2A, left images). R-SMCs displayed increased Cx43 expression compared with S-SMCs and did not express Cx40 (Figure 2A, right images). Immunofluorescence staining for Cx37 was negative in both S- and R-SMCs (data not shown). The amount of Cx43 and Cx40 was evaluated by Western blotting of S- and R-SMC extracts and confirmed results obtained by immunofluorescence staining. Cx43 antibodies recognized 2 bands migrating between 41 to 45 kDa in all cell extracts (Figure 2B). Cx43 expression was significantly increased in R-SMCs compared with S-SMCs (Figure 2C). Cx40 antibodies detected 2 bands migrating between 38 to 41 kDa in S-SMCs only (Figure 2B and 2C).
Thus, Cx expression in S- and R-SMCs matches well with the expression patterns observed in porcine CAs in vivo.

Migration of S- and R-SMCs After Inhibition of GJIC

In vitro chemotaxis assays using a modified Boyden chamber (Figure 3A) demonstrated enhanced migration \((P<0.01)\) of the R-SMCs compared with S-SMCs at both concentrations of the chemottractant PDGF-BB. Interestingly, the pharmacological gap junction blocker \(\alpha\)-GA reduced migration of R-SMCs \((P<0.05)\) but not of S-SMCs.

Microinjection of the fluorescent tracer Lucifer yellow into 1 R-SMC resulted in its diffusion to at least 4 neighboring cells (Figure 3B and 3C). Lucifer yellow diffusion in R-SMCs was significantly higher than that observed in S-SMCs \((P<0.01)\). It was markedly inhibited in R-SMCs treated with \(\alpha\)-GA \((P<0.01)\). Together, these data are consistent with the idea that reducing GJIC decreased R-SMC migration.

Effect of Selective Cx43 Inhibition on Migration of R-SMCs

As illustrated in Figure 4A, Cx43 expression was dramatically decreased in response to 100 \(\mu\)mol/L Cx43 antisense (right), whereas the same concentration of sense oligonucleotides did not affect the expression of the protein (middle).
The ability of Cx43 antisense to reduce Cx43 expression in R-SMCs was verified by Western blot. Indeed, 100 μmol/L Cx43 antisense decreased Cx43 by \( \approx 65\% \) \((P<0.05)\), whereas 100 μmol/L sense oligonucleotide had no effect (Figure 4B). Migration assays demonstrated that the downregulation of Cx43 by antisense significantly reduced PDGF-BB–induced migration of R-SMCs \((P<0.05)\). As expected, migration of R-SMCs was not affected by 100 μmol/L sense oligonucleotide or by 5 μmol/L Cx43 antisense, a concentration that also did not affect Cx43 expression. PDGF-BB–induced migration of R-SMCs was also significantly reduced by a specific Cx43 blocking peptide \((P<0.01);\) Figure 4D). Microinjection of Lucifer yellow into R-SMCs treated with Cx43 sense oligonucleotides or random peptides resulted in its diffusion to approximately 5 neighboring cells (Figure 4C and 4D). Lucifer yellow diffusion was markedly inhibited in R-SMCs treated with Cx43 antisense or Cx43 blocking peptide \((P<0.001)\). In conclusion, PDGF-BB–induced migration of R-SMCs is to a large extent prevented by inhibiting Cx43 expression or Cx43 channel function.

**Effect of Selective Cx43 Inhibition on PDGF-BB–Induced Phenotypic Change of S-SMCs**

Long-term exposure to PDGF-BB induced morphological changes in S-SMCs toward an R phenotype.\(^7\)\(^8\) We examined Cx43 and Cx40 expression during this modulation. As illustrated in Figure 5A (top images), Cx43 expression was markedly increased in S-SMCs after 4 and 7 days of PDGF-BB treatment. In contrast, Cx40 expression was decreased after 4 days of exposure to PDGF-BB and was abolished after 7 days (Figure 5A, bottom images). Differences in Cx expression levels of S-SMCs in response to the growth factor were confirmed with Western blot (Figure 5C): PDGF-BB–treated SMCs expressed 2 times more Cx43 \((P<0.05)\) and almost no Cx40 \((P<0.001)\)

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**Figure 5.** Effect of Cx43 inhibition on PDGF-BB–induced S-to-R-phenotypic change. A, Immunofluorescence staining for Cx43 (top images) and Cx40 (lower images) in green were performed on S-SMCs under control conditions and after 4 or 7 days of treatment with 10 ng/mL PDGF-BB. Cells were counterstained with Evans blue (in red). Bar=50 μm. B, Phase-contrast microphotographs showing the elongated morphology of S-SMCs under control conditions (left images) and the R-like morphology after 4 (top middle) and 7 days (bottom middle) of treatment with 10 ng/mL PDGF-BB. PDGF-BB–induced phenotypic changes of S-SMCs were prevented by coincubation with 100 μmol/L Cx43 antisense at both 4 (top right) and 7 days (bottom right). Bar=120 μm. C, Western blots (top) for Cx43, Cx40, α-SMA, and S100A4 were performed on S-SMC extracts under control conditions (left lanes) or after 7 days of treatment with 10 ng/mL PDGF-BB without (middle lanes) and in the presence of (right lanes) 100 μmol/L Cx43 antisense. C, Bottom, Bar graphs showing quantification of Western blots obtained from 3 to 6 independent experiments using S-SMCs extracts under control conditions or after 4 and 7 days of treatment with 10 ng/mL PDGF-BB without and in the presence of 100 μmol/L Cx43 antisense. \(^*P<0.05\), \(^**P<0.01\) and \(^***P<0.001\) compared with control S-SMCs, \(^#P<0.05\) and \(^##P<0.01\) compared with S-SMCs treated with PDGF-BB only.
as compared with control S-SMCs. Interestingly, PDGF-BB–induced phenotypic change of S-SMCs toward R-phenotype was prevented by 100 μmol/L Cx43 antisense. As shown in Figure 5B, S-SMCs maintained their typical elongated appearance when exposed to PDGF-BB after 4 days and to a lesser extent after 7 days of treatment with Cx43 antisense. As expected, the PDGF-BB–induced upregulation of Cx43 was largely prevented by Cx43 antisense (P<0.05; Figure 5C). In addition, the Cx43 antisense partially restored the expression of Cx40 in PDGF-BB–treated cells (Figure 5C). Furthermore, we confirmed that the expression of α-SMA, a marker of SMC differentiation, was decreased in S-SMCs after 4 and 7 days of treatment with PDGF-BB (P<0.001; Figure 5C). Addition of Cx43 antisense partially restored α-SMA expression (P<0.01). Concomitantly, S100A4, a marker of R-SMCs, was significantly upregulated after 4 and 7 days of PDGF-BB treatment (P<0.01). This upregulation was prevented by the Cx43 antisense (P<0.05).

Discussion

In the present study, we investigated the role of Cxs with respect to SMC phenotype using the porcine CA as a model. SMC populations isolated from the porcine CAs exhibit distinct phenotypes in vitro: spindle-shaped and rhomboid. S-SMCs were predominant in the normal media, whereas R-SMCs were recovered in higher proportion from stent-induced IT. In this study, we show in vitro that S- and R-SMCs have different Cx expression patterns: S-SMCs express both Cx43 and Cx40, whereas R-SMCs do not express Cx40 but express higher levels of Cx43 as compared with S-SMCs. During IT formation, growth factors produced by either a dysfunctional endothelium and/or inflammatory cells induce a SMC phenotypic modulation. We have previously shown in vitro that PDGF-BB promotes a switch from a S to an R phenotype and that this modulation was accompanied by the expression of S100A4. We show here that Cx43 is upregulated during this modulation and that avoiding PDGF-BB–induced upregulation by a Cx43 antisense prevented the S-to-R phenotypic change. In vivo, we found that Cx40 was highly expressed in the normal media, whereas Cx43 was barely detectable. In contrast, in stent-induced IT, Cx40 was downregulated and Cx43 was markedly upregulated.

Percutaneous coronary intervention is a commonly used technique to treat critically narrowed atherosclerotic blood vessels. However, its long-term efficacy is limited by restenosis or renarrowing of the arteries at the site of intervention. Restenosis is a local vascular symptom of a general biological response to injury. The stretching of a diseased artery induces an exaggerated response to injury that involves recruitment and infiltration of leukocytes to the damaged site as well as a surge in cytokines and growth factors. Subsequently, medial SMCs migrate toward the intima, where they proliferate and undergo phenotypic changes. These changes are associated with modulation of the extracellular matrix. The sum of all these events leads to IT. In the past few years, investigators have focused on the paracrine signaling mechanisms mediating the response to vascular injury, and therapeutic strategies have been developed accordingly. However, the clinically proven scope of antirestenotic agents is limited, and additional strategies are needed.

In recent years, many investigations have focused on a possible role for Cxs in the exaggerated response to vascular injury. The association between Cx43 and IT formation following balloon angioplasty has been investigated first by the group of Nicholas Severs in the rat carotid artery. These authors observed increased expression of Cx43 in intimal SMCs, which paralleled the changes in activation and phenotype of these cells. Enhanced intimal Cx43 expression was also reported in restenotic lesions of injured vessels in other species. These studies suggested that Cx43 expression levels in vascular SMCs are intimately linked to their phenotype. In agreement with this notion, it has been reported that cytokine-induced modulation of cultured SMCs from a differentiated to a dedifferentiated state coincided with more numerous and larger gap junctions as well as increased expression of Cx43. The causality in the relation between Cx43 levels and proliferation, as well as migration of SMCs, has been recently demonstrated using primary arterial SMCs obtained from Cx43 homozygous and heterozygous mice.

The potential for a Cx43-based approach to limit restenosis in vivo has been investigated in different transgenic mice. We have subjected hypercholesterolemic Cx43−/−LDLR−/− and Cx43+/+LDLR−/− mice to carotid balloon distension injury, which induced marked endothelial denudation and activation of medial SMCs. We observed restricted IT formation after balloon injury in Cx43−/−LDLR−/− mice that was associated with decreased inflammatory response and reduced SMC proliferation and migration toward the injured site. Our findings are in sharp contrast to the work of Liao et al., who studied the effects of carotid artery injury in a line of smooth muscle cell-specific Cx43 gene knockout mice (SM-Cx43 KO). Surprisingly, injury to carotid arteries in these mice produced markedly greater IT and adventitial growth than seen in control animals. Of note, SM-Cx43 KO mice were subjected to vascular occlusion or wire injury in a nonatherosclerotic context. Thus, differences between the results in the 2 studies may simply reflect different vascular adaptive processes. At any rate, these studies provide direct evidence that SM Cx43 gap junctions play an important role in modulating the in vivo growth response of vascular SMCs to acute vascular injury, making it thereby an attractive target for reducing restenosis after percutaneous coronary intervention. The relevance of other vascular Cxs in the formation of IT is presently not known. In addition, it is not clear whether the principle “reducing Cx43 limits IT formation in atherosclerotic arteries” may be extended to other mammalian species.

Of all the species examined to date, the pig is most similar to the human in its cardiovascular morphology and physiology, as well as in its susceptibility to atherosclerosis. Similar to the human situation and to an earlier study in pigs, we found Cx40 and Cx43 expression in porcine CA. Interestingly, Cx43 gap junctions are not uniformly distributed in the media of CAs. As described in detail for the rat carotid artery, higher Cx43 expression levels are observed toward the abluminal side of the media in vivo. In this study, we have used 2 distinct medial SMC populations isolated from the porcine CA. As previously described, SMCs enzymatically isolated from the normal media constantly exhibited a spindle-shaped phenotype, whereas the proportion...
of tissue explants giving rise to the 2 phenotypes depended on the orientation of the explant. When the abluminal side of the media was placed in contact with the culture dish, explants yielded a high proportion of R-SMCs and when the luminal side of the media was in contact with the culture dish relative higher proportions of S-SMCs were obtained, suggesting that intimal SMCs may be recruited from the abluminal side of the media. Whether the higher Cx43 levels observed toward the abluminal side of the media in vivo can be specifically attributed to the R-SMC subpopulation remains to be investigated.

In this study, we observed that Cx43 expression levels were upregulated and Cx40 was absent in the IT after stenting of the porcine CA. This expression pattern was retained in S-SMCs and R-SMCs. Thus, the porcine CA SMC subpopulations represent a valuable model to investigate the clinical potential of specific gap junction inhibitors in the prevention of restenosis. We observed that inhibition of Cx43 expression or channel function in R-SMCs with Cx43 antisense, α-GA, and Cx43-specific blocking peptides inhibited their migratory activity. The fact that these approaches did not affect migration in S-SMCs may possibly be explained by the very modest GJIC observed between these cells. Whether the expression of an additional Cx, ie, Cx40, in S-SMCs is responsible for their lower proliferative, migratory, or proteolytic activities remains to be investigated.

Long-term exposure to PDGF-BB induced morphological changes in S-SMCs toward a rhomboid phenotype.7,8 Interestingly, these morphological changes coincide with a loss of Cx43 expression and S-SMC toward a rhomboid phenotype could be prevented by inhibition of Cx43 expression with antisense. The maintenance of their typical elongated shape was accompanied by preservation of at least 1 SMC differentiation marker. Moreover, induction of the R-SMC marker S100A4 by PDGF-BB was also prevented. Unfortunately, we were unable to investigate whether inhibition of Cx43 channel function would be sufficient to prevent PDGF-BB–induced phenotypic modulation of S-SMCs because of aspecific adverse effects of long-term treatment with α-GA and degradation of peptides in culture medium. Thus, limiting the growth factor-induced upregulation of Cx43 avoided the change toward a deleterious SMC phenotype. Similar to our earlier studies on transgenic mice, these studies on the porcine CA model suggest that targeting Cx43 may be a promising strategy for reducing restenosis after percutaneous coronary intervention. In this respect, recent in vivo applications of Cx43 antisense gel to increase wound healing and limit burn extension in the mouse skin15,24,46 are of particular interest.

**Acknowledgments**

We thank Marc Bacchetta for excellent technical assistance.

**Sources of Funding**

This work was supported by Swiss National Science Foundation grants PPOOA-68883 and PPOOA-116897 (to B.R.K.) and 32-1165951 to (M.-L.B.-P.), the Fondation Gustave et Simone Prevoet, the Fondation Carlos and Elsie De Reuter, and the Swiss University Conference Program Heart Remodeling in Health and Disease.

**Disclosures**

None.

**References**


