Knockdown of connexin 43 attenuates balloon injury-induced vascular restenosis through the inhibition of the proliferation and migration of vascular smooth muscle cells

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Abstract. Coronary artery disease (CAD) or atherosclerotic heart disease is one of the most common types of cardiovascular disease. Although percutaneous coronary intervention [PCI or percutaneous transluminal coronary angioplasty (PTCA)] is a mature, well-established technique used to treat atherosclerotic heart disease, its long-term therapeutic effects are compromised by a high incidence of vascular restenosis (RS) following angioplasty. In our previous study, we found that the principal gap junction protein, connexin 43 (Cx43), in vascular smooth muscle cells (VSMCs) was involved in the development of vascular RS following angioplasty-induced balloon injury. However, the exact role action of Cx43 in vascular RS remains unclear. In the present study, we aimed to further examine whether the knockdown of Cx43 attenuates the development of vascular RS through the inhibition of the proliferation and migration of VSMCs. We found that the use of a lentiviral vector expressing shRNA targeting Cx43 (Cx43-RNAi-LV) efficiently silenced the mRNA and protein expression of Cx43 in cultured VSMCs. In addition, MTT and Transwell assays were used to examine the proliferation and migration of the VSMCs, respectively. The results revealed that the knockdown of Cx43 by Cx43-RNAi-LV at a multiplicity of infection (MOI) of 100 significantly inhibited the proliferation and migration of the VSMCs in vitro. Notably, the knockdown of Cx43 also effectively attenuated the development of vascular RS and intimal hyperplasia following balloon injury in vivo. Taken together, our data suggest that Cx43 is involved in the development of vascular RS and intimal hyperplasia through the regulation of the proliferation and migration of VSMCs. Thus, the present study provides new insight into the pathogenesis of vascular RS, and suggests that further confirms that Cx43 may well be a novel potential pharmacological target for preventing vascular RS following PCI.

Introduction

Coronary artery disease (CAD) or atherosclerotic heart disease is one of the most common types of cardiovascular disease with a high incidence of morbidity and mortality. It is well acknowledged that atherosclerosis is the main cause of CAD. Of the therapeutic measures currently available, percutaneous coronary intervention [PCI or percutaneous transluminal coronary angioplasty (PTCA)] has developed into a mature, well-established technique used to treat CAD since it was first introduced in 1977 (1). PCI effectively improves coronary blood flow, reduces angina pectoris and greatly improves the quality of life of patients (2). Another advantage of PCI is that it is a relatively easy technique and is minimally invasive. However, its long-term therapeutic effects are often compromised by the high incidence of vascular restenosis (RS) following angioplasty. In clinical practice, the incidence of RS is approximately 20-50% at 6 months post-PCI and even with stent implantation, the incidence of RS is also approximately 25-30% (3). Therefore, vascular RS is a serious complication of PCI, and it is neces-
sary to fully elucidate the molecular mechanisms responsible for the development of RS following angioplasty so that a novel therapeutic methods can be found to prevent the occurrence of vascular RS following angioplasty.

It has been suggested that the proliferation and migration of vascular smooth muscle cells (VSMCs) may be key factors involved in the development of RS following angioplasty (4). Mature VSMCs are highly differentiated, and they are principally responsible for contraction. However, VSMCs also proliferate and produce the matrix components of the blood vessel wall under specific pathophysiological conditions, such as vasculogenesis (4). Moreover, VSMCs still retain remarkable plasticity, and may undergo relatively rapid and reversible changes in their phenotype in response to local environmental stress (5). Vascular RS at an early stage, often occurs several hours or several days following PCI, and RS is mainly induced by vasoospasm or decreased vessel elasticity. Subsequently, some cytokines, including endothelin, angiotensin II, basic fibroblast growth factor, platelet-derived growth factor and transforming growth factor are released from damaged endothelial cells and macrophages which have invaded the endothelial sublayer. These cytokines stimulate the migration and proliferation of VSMCs, and induce the accumulation of extracellular matrix components, which leads to the remodeling of the blood vessel wall and ultimately to vascular RS (6). Although some intracellular signaling pathways have been found to regulate the proliferation and migration of VSMCs (2,7,8), the role of direct intercellular communication pathways and gap junction (GJ) channels in the proliferation and migration of VSMCs, as well as their contribution to RS still requires further investigation.

GJs are direct intercellular communication pathways, which consist of assemblies of channel proteins known as connexins (9). Ions, second messengers and small metabolites of up to 1 kDa in molecular mass may be rapidly exchanged through GJs (10). However, the precise assembly of their component connexins influences the properties of GJ channels, including permeability and conductance (11). In arteries, the endothelium expresses three connexin isoforms, connexin (Cx)40, Cx37 and Cx43. By contrast, VSMCs predominantly express Cx43 and, in some instances, Cx40 or Cx45 (12-14). It has been reported that the expression of Cx43 is upregulated during the alteration of the VSMC phenotype (15). Moreover, Cx43 remodeling involving changes in size, structure, quantity and distribution may also occur in vascular lesions (16). Cx43 remodeling influences not only the conductivity and permeability of the GJ itself, but also the electrical, chemical and metabolic channels between adjacent cells (17-19). In our previous study, we found that Cx43 was involved in the development of vascular RS following angioplasty-induced balloon injury (20). However, the underlying mechanisms remain unclear.

In the present study, we further examined whether the knockdown of Cx43 effectively attenuated the development of vascular RS following balloon injury in vivo. Our data further indicate that Cx43 plays a role in the development of vascular RS and intimal hyperplasia by regulating the proliferation and migration of VSMCs.

Materials and methods

Experimental animals. Male Sprague-Dawley rats (purchased from the Department of Animal Science, Nanchang University, Nanchang, China) weighing 300-400 g were maintained on a regular chow diet prior to the study. All animal experimental procedures were in accordance with the current National Institutes of Health Guidelines, and they were approved by the Ethics Committee for Animal Experiments at Nanchang University.

Cell culture. The culture of the VMSCs was performed as previously described (21). Briefly, the rats were anesthetized with an intraperitoneal injection of Hydar (10%, 3.5 ml/kg) and Harbin Pharmaceutical Group Co., Ltd., Harbin, China) and the carotid artery was quickly removed under aseptic conditions. After stripping the extravascular connective tissue, the vessels were longitudinally cut off and the internal membrane was positioned upward in a glass culture dish. Endothelial denudation was achieved by digestion with 0.25% trypsin. The tunica media vasmor was cut into 1-mm²-sized tissue blocks. The internal membrane was positioned upward in a plastic culture dish. These tissue blocks were incubated at 37°C in a 5% CO₂ saturated humidity incubator for 1-2 h and submerged in 2 ml medium after firm adhesion. After 5-7 days, the VSMCs had migrated from the tissue blocks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Trans Serum™; Transgen Biotech, Beijing, China) and 1% penicillin/streptomycin (P/S; Solarbio, Beijing, China). Third to fifth generation VSMCs were used in the experiments.

Construction of GFP-Cx43-shRNA-lentiviral vectors. To examine the role of Cx43 in vascular RS, a lentiviral vector expressing shRNA targeting Cx43 (Cx43-RNAi-LV) was constructed (GeneChem, Shanghai, China). Briefly, the shRNA sequence for Cx43 (5'-AGAGCACGGCAAGGTGAAA-3') was designed using the manufacturer's RNA interference (RNAi) designer program, and the negative control construct (control shRNA) was created using a scrambled sequence (5'-TTTCCAGACGTCAGTCT-3'), as described in a previous study (22). DNA oligos were chemically synthesized (GeneChem), annealed and inserted into the expression vector by double digestion with AgeI and EcoRI (New England Biolabs, Ipswich, MA, USA), and ligated with T4 DNA ligase (TakaRa, Dalian, China) in accordance with the manufacturer's instructions. The ligation was transformed into competent E. coli cells and confirmed by restriction enzyme analysis and DNA sequencing. The sequences were then cloned into pGCSIL-GFP to generate lentiviral vectors. The expression vectors and package vectors were transfected into 293T cells (ATCC, Manassas, VA, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h of culture, the supernatants containing lentiviruses, such as Cx43-RNAi-LV and NC-GFP-LV (negative control) were harvested.
Purification was then performed using ultracentrifugation and the lentiviral titer was calculated (as 10^7 TU/µl).

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from the rat carotid tissue using TRIzol reagent (Tiangen, Beijing, China), and cDNA was synthesized from the extracted total RNA using the SuperScript III kit (Promega, Madison, WI, USA) following the manufacturer's instructions. The specific primers for PCR were as follows: Cx43 sense, 5'-AAAGGCGTTAAGGATCGCGTG-3' and antisense, 5’-GTCATCAAGCCGGAGGCTCT-3' (23); β-actin sense, 5’-CCCATCTATGGAGGGTTACGC-3' and antisense, 5’-TTTATGTCACGCACAGTTTC-3’. All specific primers were chemically synthesized (Generay Biotech Co. Ltd., Shanghai, China). The PCR reactions were performed using the GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The amplification conditions for Cx43 were as follows: 94°C for 2 min followed by 32 cycles at 94°C for 45 sec, 58°C for 45 sec, and 72°C for 90 sec, and the final extension at 72°C for 5 min. The PCR conditions for β-actin were as follows: 94°C for 2 min followed by 28 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 90 sec, and the final extension at 72°C for 5 min. The amplified RT-PCR products were separated on 1.2% (w/v) agarose containing ethidium bromide (both from Sangon Biotech). The whole cell lysate was resuspended in sample buffer containing 4% sodium dodecyl sulfate (SDS; Sangon Biotech). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% skim milk; Chemicon, Temecula, CA, USA) overnight at 4°C. The membranes were then washed 3 times with TBST, and then incubated with the relative HRP-conjugated IgG secondary antibody (BV-S8008; Zhongshan Golden Bridge Biotechnology Co., Beijing, China) for 2 h at room temperature, and washed in TBST 3 times. A chemiluminescence assay was carried out with Amersham ECL Prime Western Blotting Detection reagents, and the immunoblotting signal was detected using the Molecular Imager ChemiDoc™ XRS+ system (Bio-Rad). The intensity of each Cx43 band was detected using the Molecular Imager Blotting Detection reagents, and the immunoblotting signal as follow: 94°C for 2 min followed by 28 cycles at 94°C for 30 sec, 56°C for 30 sec, and 72°C for 90 sec, and the final extension at 72°C for 5 min. The amplified RT-PCR products were separated on 1.2% (w/v) agarose containing ethidium bromide (both from Sangon Biotech). The whole cell lysate was resuspended in sample buffer containing 4% sodium dodecyl sulfate (SDS; Sangon Biotech). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blocked in 5% skim milk; Chemicon, Temecula, CA, USA) overnight at 4°C. The membranes were then washed 3 times with TBST, and then incubated with the relative HRP-conjugated IgG secondary antibody (BV-S8008; Zhongshan Golden Bridge Biotechnology Co., Beijing, China) for 2 h at room temperature, and washed in TBST 3 times. A chemiluminescence assay was carried out with Amersham ECL Prime Western Blotting Detection reagents, and the immunoblotting signal was detected using the Molecular Imager ChemiDoc™ XRS+ system (Bio-Rad). The intensity of each Cx43 band was normalized to the GAPDH band, and the relative expression of Cx43 following viral infection was normalized to the control.

MTT assay. MTT assay was applied to assess the proliferation of the VSMCs according to the manufacturer’s instructions. Briefly, 5x10^4 cells were seeded in a 96-well tissue culture plates and cultured in growth medium. The VSMCs were infected with NC-GFP-LV or Cx43-RNAi-LV at an MOI 0.50, 100, 150 and 200. At 24, 48, 72 and 96 h after viral infection, 20 µl of MTT solution were added to each well (5 mg/ml, 0.5% MTT) and the cells were cultured for a further 4 h to detect the proliferation of the VSMCs. Subsequently, 100 µl of dimethyl sulfoxide were added to each well, and the culture plate was shaken at a low speed for 10 min until the crystals dissolved completely. The color intensity was measured spectrophotometrically using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Waltham, MA, USA) at 492 nm. All assays were performed at least 3 times.

Transwell assay. Transwell assays were performed with Transwell chambers (Corning Inc., Corning, NY, USA) as previously described (24). Briefly, 5.0x10^4 VSMCs were seeded into the upper chamber with 200 µl of serum-free medium following infection with NC-GFP-LV or Cx43-RNAi-LV at an MOI of 100. The bottom of the upper chamber was incubated in 500 µl of complete medium containing 10% fetal calf serum and 1% P/S. Following 12 h of incubation, the cells on the top surface of the insert were gently removed with a cotton swab. The migrated cells on the lower surface were fixed with 4% paraformaldehyde and stained with crystal violet (both from Sigma-Aldrich, St. Louis, MO, USA) for 30 min. The migrated cells were photographed and counted in 4 random fields.

Establishment of model of vascular RS induced by balloon injury. The rats were anesthetized with an intraperitoneal injection of Hydral (10%, 3.5 ml/kg; Harbin Pharmaceutical Group Co., Ltd.). To establish a model of vascular RS, the angioplasty balloon (1.5x20 mm; Cordis Corp., Miami, FL, USA) was inserted into the rat common carotid artery through an incision in the left external carotid artery as previously described (25). The balloon was then inflated sufficiently in the carotid artery and was drawn 3 times consistently from proximal area to the carotid bifurcation to produce endothelial denudation. The external carotid was ligated and blood flow in the common carotid was restored. To examine the effects of Cx43-RNAi-LV on balloon injury-induced vascular RS, the rats were randomly divided into 3 groups (n=6/group): control group (no balloon injury); injury group (balloon injury only) and Cx43-RNAi-LV-treated injury group (balloon injury + Cx43-RNAi-LV). The lentivirus (5x10^5 TU/ml, 100 µl) expressing shRNA was injected into the balloon-injured rat carotid arteries using a polyethylene catheter, to knockdown Cx43 in the VSMCs. Benzylpenicillin sodium (40x10^4 IU/day for 3 days; Harbin Pharmaceutical Group Co., Ltd.) was then administered by intramuscular injection to prevent infection. All the rats were euthanized by an overdose of Hydral at 28 days following balloon injury. Three serial cryosections (5 µm thick) were prepared from the middle portion of the rat common carotid arteries. The slices were stained with hematoxylin and eosin (H&E; Sangon Biotech, Shanghai, China), and histomorphologic observation was performed under a light microscope (Olympus, Tokyo, Japan) to examine the structure of the blood vessel wall following balloon injury. The intimal and medial area of 3 serial cryosections from each sample was measured using Image-Pro Plus 5.0 software (Media Cybernetics, Inc., Houston, TX, USA) in order to evaluate vascular remodeling.
Statistical analysis. The quantitative data are presented as the means ± standard deviation (SD). Data were analyzed using the Student's t-test to compare two conditions, and a value of P<0.05 was considered to indicate a statistically significant difference.

Results

Lentivirus-mediated knockdown of Cx43 in cultured VSMCs.

In our previous study, it was found that Cx43 is involved in the development of vascular RS and intimal hyperplasia after balloon injury (20). As the intimal hyperplasia and vascular RS which occur following balloon injury are mainly induced by the proliferation and migration of VSMCs, we used a lentiviral vector expressing shRNA targeting Cx43 (Cx43-RNAi-LV) to silence Cx43 in the cultured VSMCs. The results of RT-PCR revealed that the mRNA level of Cx43 was significantly decreased in the VSMCs infected with Cx43-RNAi-LV, but not in the control (uninfected) or the NC-GFP-LV-infected cells. Compared with the controls, the mRNA level of Cx43 in the Cx43-RNAi-LV-infected VSMCs decreased to approximately 54.3% (Fig. 1A). Moreover, the protein expression of Cx43 in the VSMCs was examined by western blot analysis. Compared with the controls, the protein level of Cx43 in the VSMCs infected with Cx43-RNAi-LV decreased to approximately 49.4% (Fig. 2). These results indicated that the lentivirus-mediated knockdown of Cx43 in the cultured VSMCs was achieved by infection with Cx43-RNAi-LV.

Effect of knockdown of Cx43 on the proliferation of cultured VSMCs.

Intimal hyperplasia and vascular RS which occur following balloon injury are mainly induced by the proliferation and migration of VSMCs, and the principal gap junction protein in VSMCs, Cx43, plays an important role in the development of intimal hyperplasia and vascular RS (4). Thus, to examine the role of Cx43 in the proliferation of VSMCs, Cx43-RNAi-LV was used to specifically silence Cx43 in the VSMCs, and the lentiviral vector, NC-GFP-LV, was used as the negative control. The proliferation of the VSMCs under each condition was evaluated by MTT assay. Infection with Cx43-RNAi-LV at an MOI of 100, 150 and 200 significantly inhibited the proliferation of the VSMCs at 24, 48, 72 and 96 h following
infection (Fig. 3). By contrast, infection with NC-GFP-LV and Cx43-RNAi-LV at an MOI of 50 had no significant effect on the proliferation of the VSMCs. The results of MTT assay indicated that the gap junction protein, Cx43, is important for the proliferation of VSMCs.

Knockdown of Cx43 effectively inhibits the migratory activity of the VSMCs. In addition, the migration of the VSMCs is involved in the development of intimal hyperplasia and vascular RS (4). Thus, to examine the role of Cx43 in the migration of VSMCs, Cx43 in the VSMCs was specifically silenced by Cx43-RNAi-LV. The migratory activity of the VSMCs in which Cx43 was knocked down or not was evaluated by Transwell assay. Compared with the control (uninfected) or the negative control (NC-GFP-LV), the knockdown of Cx43 by Cx43-RNAi-LV significantly attenuated the migration of the VSMCs (Fig. 4). Taken together, the results of MTT assay and Transwell assay, provide direct evidence that Cx43 is a vital regulatory factor in the proliferation and migration of VSMCs. Our results also suggest that Cx43 is involved in the develop-
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ment of intimal hyperplasia and vascular RS which occur following balloon injury, by regulating the proliferation and migration of VSMCs.

Knockdown of Cx43 attenuates the development of intimal hyperplasia and restenosis (RS) induced by balloon injury in vivo. Since the proliferation and migration of VSMCs is important in the development of intimal hyperplasia and vascular RS following balloon injury, and the knockdown of Cx43 effectively attenuated the proliferation and migration of the VSMCs (Figs. 3 and 4), we further examined whether the knockdown of Cx43 using Cx43-RNAi-LV prevents the development of intimal hyperplasia and vascular RS following balloon injury in vivo. The histomorphological observations of the arterial sections (Fig. 5A) and statistical analysis of the intimal/medial areas (Fig. 5B) revealed that the lentivirus-mediated knockdown of Cx43 by Cx43-RNAi-LV significantly attenuated the development of balloon injury-induced intimal hyperplasia and vascular RS. These results are consistent with the results of MTT assay and Transwell assay in vitro, and suggest that Cx43 is involved in the development of intimal hyperplasia and vascular RS following balloon injury, through the regulation of the proliferation and migration of VSMCs.

Discussion

Vascular RS and intimal hyperplasia are the main adverse effects of PCI, which usually limit the clinical effects of PCI. Evidence indicates the vital role of endothelial denudation, as well as the proliferation and migration of VSMCs in vascular RS and intimal hyperplasia (1,2). The related molecular mechanisms, nevertheless, remain unclear. On the other hand, studies have suggested the involvement of GJs in the development of some vascular diseases, such as hypertension, atherosclerosis and RS (24,26). Particularly, the principal GJ protein, Cx43, in VSMCs may be involved in the development of vascular RS and intimal hyperplasia (20,27). In transgenic mice, it was found that a reduced Cx43 expression effectively inhibited acute neointimal formation in hypercholesterolemic mice (28). In our previous study (20), we also found that the expression of the GJ protein, Cx43, was upregulated in vessels following balloon injury. The knockdown of Cx43 effectively prevented

Figure 5. Knockdown of connexin 43 (Cx43) attenuates the development of intimal hyperplasia and restenosis (RS) induced by balloon injury. (A) Histomorphological observations of the arteries. Carotid arteries were removed after the indicated treatments and they were cut into sections (5-µm-thick) for hematoxylin and eosin (H&E) staining and subsequent examination under a microscope. Images at low and high magnification are shown in the upper and lower panels, respectively (upper panel, x100 magnification; bottom panel, x400 magnification). The normal artery without balloon injury is shown in the left panel (control). The artery after balloon injury is shown in the middle panel. The artery treated with Cx43-RNAi after balloon injury is shown in the right panel. (B) The intimal and medial area of the arterial sections was measured using Image-Pro Plus 5.0 software. Data represent the means ± SD of 6 independent experiments. **P<0.01.
balloon injury-induced vascular RS and intimal hyperplasia (20). Moreover, evidence suggests that GJs are involved in the proliferation and migration-related signaling pathways of VSMCs (22,29). The blockade of Cx43 hemichannels has been shown to reduce neointima formation by inhibiting the proliferation and phenotypic modulation of VSMCs (30). Cx43 is also involved in the angiotensin II- or oxidized-phospholipid-induced migration and proliferation of VSMCs (8,31,32). However, direct evidence is still lacking as to the role of Cx43 in the proliferation and migration of VSMCs induced by endothelial denudation or balloon injury. Therefore, it is interesting to investigate the direct effects of Cx43 silencing on the proliferation and migration of VSMCs and the development of vascular RS following PCI.

In the present study, we constructed a lentiviral vector expressing shRNA targeting Cx43, Cx43-RNAi-LV, to silence Cx43. Cx43-RNAi-LV effectively knocked down Cx43 in the cultured VSMCs (Figs. 1 and 2). These results are consistent with the in vivo results of our previous study (20). To examine the effects of Cx43-RNAi-LV on the proliferation of VSMCs, the cells were infected with Cx43-RNAi-LV at various MOIs (0, 50, 100, 150 and 200). Compared with infection at an MOI of 0 or infection with the negative control, infection with Cx43-RNAi-LV at MOIs of 100, 150 and 200 significantly inhibited the proliferation of VSMCs (Fig. 3). In addition, the migration of the cultured VSMCs under each experimental condition was evaluated by Transwell assay. Infection with Cx43-RNAi-LV at an MOI of 100 significantly attenuated the migratory activity of the VSMCs (Fig. 4). By contrast, infection with NC-GFP-LV had no significant effect on the migration of the VSMCs. These in vitro results are consistent with those of a previous study (30), and provide direct evidence that Cx43 plays an important role in regulating the proliferation and migration of VSMCs. On the other hand, the lentivirus-mediated knockdown of Cx43 effectively attenuated the development of vascular RS and intimal hyperplasia following balloon injury (Fig. 5). Taken together, these results indicate that Cx43 contributes to the development of vascular RS and intimal hyperplasia through the regulation of the proliferation and migration of VSMCs. Although the detailed mechanisms involved remain unclear, it has been suggested the MAPK signaling pathway regulates the role of Cx43 in the proliferation and migration of VSMCs (8,33). The MAPK-AP-1 signaling pathway has been found to regulate the role of Cx43 in the angiotensin II-induced migration and proliferation of saphenous vein smooth muscle cells (8). Moreover, the MAPK-mediated phosphorylation of Cx43 promotes the proliferation of VSMCs through the facilitation of the binding of Cx43 to cell cycle protein cyclin E (33).

In conclusion, our data demonstrate that the knockdown of the principal GJ protein in VSMCs, Cx43, may be an effective measure to prevent the development of intimal hyperplasia and vascular RS through the inhibition of the proliferation and migration of VSMCs. In the present study, we constructed a lentiviral vector expressing shRNA targeting Cx43. Infection with Cx43-RNAi-LV effectively silenced the mRNA and protein expression of Cx43 in the VSMCs. In addition, the knockdown of Cx43 with Cx43-RNAi-LV significantly inhibited the proliferation and migration of VSMCs in vitro, and effectively attenuated the development of vascular RS and intimal hyperplasia following balloon injury in vivo. These results demonstrate that the GJ protein, Cx43, plays an important role in the development of vascular RS and intimal hyperplasia through the regulation of the proliferation and migration of VSMCs. Thus, our data suggest that Cx43 may be a novel and promising pharmacological target for preventing the development of intimal hyperplasia and RS following PCI.

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