The Cav3.1 T-type calcium channel is required for neointimal formation in response to vascular injury in mice

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Aims
Restenosis is an undesirable consequence following percutaneous vascular interventions. However, the current strategy for preventing restenosis is inadequate. The aim of this study was to investigate the role of low-voltage gated T-type calcium channels in regulating vascular smooth muscle cell (VSMC) proliferation during neointimal formation.

Methods and results
Wire injury of mice carotid arteries resulted in neointimal formation in the wild-type and Cav3.2−/− but not Cav3.1−/− mice, indicating a critical role of Cav3.1 in neointimal formation. In addition, we found a significant increase of Cav3.1 mRNA and protein in injured arteries. Cav3.1 knockout or knockdown (shCav3.1) reduced VSMC proliferation. Since T-channels are expressed predominantly in the G1 and S phases in VSMCs, we examined whether an abnormal G1/S transition was the cause of the reduced cell proliferation in shCav3.1 VSMCs. We found a disrupted expression of cyclin E in shCav3.1 VSMCs, and calmodulin agonist CALP1 partially rescued the defective cell proliferation. Furthermore, we demonstrated that infusion of NNC55-0396, a selective T-channel blocker, inhibited neointimal formation in wild-type mice.

Conclusion
Cav3.1 is required for VSMC proliferation during neointimal formation, and blocking of Cav3.1 may be beneficial for preventing restenosis.

Keywords
T-type calcium channels • Cell proliferation • Neointimal formation • Calcium channel blocker

1. Introduction
Restenosis, caused by neointimal formation, is a common adverse effect of percutaneous vascular interventions, such as balloon angioplasty and stent implantation for coronary and peripheral artery diseases. Restenosis is the result of inflammatory cell infiltration followed by vascular smooth muscle cell (VSMC) migration and proliferation at the site of vascular intervention.1–3 However, the prevention of restenosis using anti-inflammatory drugs has proven unsatisfactory in many clinical trials.4–7 Therefore, understanding the migration and proliferation of VSMCs during neointimal formation may be beneficial in identifying novel molecular targets, and thereby in reducing the frequency and severity of restenosis.

Extracellular Ca2+ influx is known to trigger cell-cycle progression and cell proliferation in many cell types including VSMCs.8 In rat VSMCs, a coordinated elevation of the intracellular Ca2+ concentration ([Ca2+]i) is required for insulin-like growth factor-1 (IGF-1)-dependent G1- to S-phase cell-cycle progression.9 In VSMCs, extracellular Ca2+ entry is mainly through two types of voltage-gated Ca2+ channels, L-type (L-channels) and T-type (T-channels). Moreover, L- and T-channels are believed to contribute to the specificity of the diverse effects of Ca2+.10,11 For example, Ca2+ influx via L-channels is required for smooth muscle contraction, and L-channel blockers are clinically used to lower blood pressure in hypertensive patients.12 On the other hand, in rat VSMCs, T-channels are predominantly expressed in the G1 and S phases, but not in the G0.
were generated and genotyped as described previously.26,27 In this study, we conducted a carotid artery wire injury model in wild-type, Cav3.1 knockout (Cav3.1−/−), and Cav3.2 knockout (Cav3.2−/−) mice, combined with an RNA interference (RNAi) approach to test our hypothesis that Cav3.1 is required for neointimal proliferation and reduce neointimal formation is lacking. In vivo evidence of specific T-channel blockers to prevent VSMC proliferation and reduce neointimal formation after PCI by reducing the rate of high-grade restenosis.21 Verapamil blockade of Cav3.1 inhibits cell proliferation of human pulmonary PASMCs.18 However, the involvement of Cav3.2 in VSMC proliferation has not yet been demonstrated.

Recent clinical trials have found that calcium channel blockers (CCBs) are probably effective in the reduction of restenosis and related clinical events after percutaneous coronary interventions (PCI).19–22 Although the currently available CCBs are mostly aimed at blocking L-channels, most of them can also partially block T-channels. For example, verapamil, an L-channel blocker, is able to improve long-term clinical outcomes of native coronary arteries after PCI by reducing the rate of high-grade restenosis.23 Verapamil also exhibits the potency to partially block T-channels.24 Furthermore, mibebradil, an L- and T-channel blocker, has a stronger anti-proliferative effect on VSMCs both in vitro and in vivo.25 Nevertheless, in vivo evidence of specific T-channel blockers to prevent VSMC proliferation and reduce neointimal formation is lacking.

In this study, we conducted a carotid artery wire injury model in wild-type, Cav3.1 knockout (Cav3.1−/−), and Cav3.2 knockout (Cav3.2−/−) mice, combined with an RNA interference (RNAi) approach to test our hypothesis that Cav3.1 is required for neointimal formation, and that T-channel blockers can thus reduce neointimal formation after artery injury. We found that Cav3.1 regulated VSMC proliferation via a calmodulin (CaM)-dependent pathway. Furthermore, blocking of Cav3.1 was beneficial in preventing neointimal formation, and hence in reducing vascular restenosis.

2. Methods

2.1 Animals

All research conformed to the National Institutes of Health and Institutional Animal Care guidelines, and also those of the Utilization Committee, Academia Sinica (Taipei, Taiwan). Cav3.1−/− and Cav3.2−/− mice were generated and genotyped as described previously.26,27

2.2 Carotid artery injury operation, tissue harvesting, and processing

Eight to 10-week-old adult male C57BL/6 mice (n = 7 each group) were anesthetized (vaporized 1% isoflurane) and received right common carotid artery injury by inserting and removing a 0.014-in. guide wire (1003315H, Abbott Vascular Inc.) three times. A typical operation procedure took < 30 min. BrdU (50 mg/kg, B5002, Sigma) was injected intra-peritoneally on Day 10, Day 11, Day 12, and Day 13 after artery injury. Two weeks after artery injury, the mice were anesthetized and perfused transcardially with phosphate buffer saline (PBS), followed by 10% neutral buffered formalin solution. Both the right and left carotid arteries were harvested and the left carotid arteries were used as negative controls. For cryosections, the mice received only PBS perfusion. The bilateral carotid arteries were isolated separately, fixed in tissue freezing medium and stored at −80°C.

2.3 Histological, immunohistochemical, and immunofluorescent analyses

Four cross-sections (5 μm thickness, 400–500 μm apart) of the injured arteries were taken for histomorphological analysis. Digitized images of the H & E and elastic stains (Elastic Stain Kit, HT25A, Sigma) were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, USA) to calculate the intimal area to the medial area ratio (IMA). Immunohistochemical stains for endothelium, VSMCs, pan-leucocytes, and the proliferative cells were conducted using anti-von Willebrand factor (vWF) antibody (1:200, A0082, DAKO), anti-smooth muscle α-actin (1:3000, A5228, Sigma), anti-mouse CD45 (1:100, 550539, BD Pharmingen), and anti-BrdU antibodies (1:400, Clone BU-33, Sigma), respectively. Total cell nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI, 5 μmMol/L in PBS, 32670, Sigma). Vascular injury was judged by the loss or disruption of endothelial cells and loosening of media. The degree of vascular injury was quantified using the ratio of total injured length to vascular inner circumference. Proliferation indices were calculated as BrdU-positive cell number/total DAPI-positive cell number in neointima and media. Anti-Ca3.1 (1:100, ACC-021, Alomone) and anti-α-actin antibodies (1:1000, A5228, Sigma) were used for immunofluorescent staining of the cryosections of mouse carotid arteries.

2.4 VSMC cultures and proliferation assays

Mouse and rat thoracic aortic VSMCs were isolated and cultured as described previously.28,29 The first passage of mouse VSMCs and passages 3–8 of rat VSMCs were used in our study. VSMCs were starved for 48 h before 10% foetal bovine serum (FBS) stimulation. For cell proliferation assay, cells were seeded at a density of 1500 cells/cm2. The cell proliferation rate was measured using a CyQUANT proliferation assay (C35007, Invitrogen) according to the manufacturer’s instructions. In some experiments, the cells were counted using a hemocytometer to validate the results of the CyQUANT proliferation assay. Calmodulin agonist, CALP1 (10 μMol/L, 2090, Towcy) was added 1 day after 10% FBS stimulation. T-type Ca2+ channel blocker, NNCS5-0396 (5 μMol/L, 2268, Tocris) was added at the same time as 10% FBS stimulation. 0.9% saline was used as the control.

2.5 Cell-cycle analysis by propidium iodide staining and flow cytometry

Trypsinized VSMCs were resuspended in PBS (1 × 106 cells/mL) and fixed with cold 70% ethanol at 4°C for at least 1 h. After fixation, the cells were resuspended in 1 mL propidium iodide (PI) solution (40 μg/mL, PI, 0.1 mg/mL RNase A, 0.05% Triton X-100 in 1 mL PBS) in the dark at 37°C for 40 min. The cells were washed and resuspended in PBS for flow analysis using a BD FACS Canto flow cytometer (BD Biosciences). We used FlowJo Windows version 7.6 (Tree Star) for the cell-cycle analysis.

2.6 Leucocyte recruitment and functional assays

A thioglycollate-induced peritonitis model was used to obtain leucocytes from the peritoneal cavity of wild-type and Cav3.1−/− mice. Cultured leucocytes were stimulated with lipopolysaccharide (LPS, 10 μg/mL) for 24 h. The culture media were collected and the amounts of TNF-α and IL-1β were determined using commercial ELISA kits.

2.7 Reverse transcription and real-time quantitative polymerase chain reaction

Total RNA was extracted from homogenized lysates of the carotid arteries, leucocytes, and cultured VSMCs using Trizol reagent (Invitrogen) according to the manufacturer’s protocols. The RNA from four arteries was pooled together for one set of TaqMan RT-qPCR experiments. The primers and probes used for detecting mouse and rat Ca3.1 and GAPDH are listed in the Supplementary material online. Because the expression of T-channels

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is cell-cycle dependent, we used double thymidine block to synchronize the rat VSMCs at the G1/S boundary for the detection of CaV3.1.

2.8 Western blot
Equal loading of 30 μg protein extracts from rat VSMCs at different time points after 10% FBS stimulation were separated by 12% SDS–PAGE for western blot. Anti-cyclin E1 antibody (1:500, GTX27959, GeneTex) was used for immunoblotting of cyclin E and anti-GAPDH antibody (1:5000, NB300-221, Novus Biologicals) for the housekeeping protein.

2.9 Lentiviral shRNA production and transduction
Lentiviral shRNA was obtained from the National RNAi Core Facility Platform of Academia Sinica, Taiwan. The methods for the production and transduction are provided at the following website (http://rnaigened.med.sinica.edu.tw/index). The shRNA sequences for Cacna1g (shCaV3.1) and luciferase (shLuc) are listed in the Supplementary material online. Rat VSMCs (2 × 10⁶ cells/well) were transfected with lentiviral particles (MOI = 5) using a spinning infection method. Puromycin (2.5 μg/mL, P8833, Sigma) was used to select lentivirus-infected cells.

2.10 NNC55-0396 dihydrochloride infusion
NNC55-0396 dihydrochloride was dissolved in 0.9% saline. NNC55-0396 (1 mg) was delivered subcutaneously for 1 week using an osmotic mini-pump (ALZET, model 1007D, Durect) implanted after the carotid injury. Saline was used as the control.

2.11 Statistics
Data are presented as mean±SEM. Statistical comparisons were performed using independent-samples t-tests for two sample groups, one-way ANOVA for three sample groups, or repeated measures ANOVA for the proliferation assay. Dunnett’s t-test was used for the post-hoc analysis with the wild-type group as the controls. A P-value of <0.05 was considered statistically significant.

3. Results
3.1 Impaired neointimal formation in CaV3.1−/− but not CaV3.2−/− mice after carotid artery injury
To investigate the involvement of T-channels in VSMC proliferation during neointimal formation in vivo, we performed carotid artery wire injuries in wild-type, CaV3.1−/−, and CaV3.2−/− mice (Figure 1A). Two weeks after carotid artery injury, neointimal formation was observed in the wild-type and CaV3.2−/−, but not in CaV3.1−/− injured carotid arteries. The I/M ratio was significantly reduced in the injured carotid arteries from CaV3.1−/− mice but not in those from wild-type and CaV3.2−/− mice (Figure 1B). These results suggest that CaV3.1, but not CaV3.2, may be involved in neointimal formation after vascular injury. In addition, since there was no significant difference in I/M ratio between the wild-type and CaV3.2−/− mice, only CaV3.1−/− mice were used for further analysis.

We next determined whether VSMCs are the major cell type responsible for the increased neointima region. Injured arteries were stained with α-actin antibody and the results showed that VSMCs were the predominant cell type in the neointima (Figure 1A). To ensure the consistency of arterial injury during surgery, H & E staining and vWF (an endothelium marker) immunohistochemical staining were performed 24 h after the wire injury (Figure 1C). Quantitative results showed that the degree of carotid artery injury was similar between wild-type and CaV3.1−/− mice (Figure 1D).

Previous studies have shown that after artery injury, a reduced inflammation response, and especially reduced leucocyte recruitment, can lead to the reduction of neointimal formation. To clarify whether the reduced neointimal formation observed in CaV3.1−/− mice was due to reduced leucocyte recruitment, we examined leucocyte recruitment by staining the injured arteries with anti-CD45 antibody, a pan-leucocyte marker, 7 days after the wire injury (Figure 1E). As shown in Figure 1E, similar numbers of CD45-positive cells were observed on the lining of the lumen side of wild-type and CaV3.1−/− injured arteries, but not on the uninjured arteries. Quantitative results showed no significant difference in leucocyte recruitment between wild-type and CaV3.1−/− mice (Figure 1F). Even though the quantities of recruited leucocytes were similar between wild-type and CaV3.1−/− mice, the function of the leucocytes may have been different or altered. To investigate this possibility, we first determined whether CaV3.1 was expressed in the leucocytes, and we found that CaV3.1 transcripts were not detectable in either wild-type or CaV3.1−/− leucocytes (see Supplementary material online, Figure S1). The responses of leucocytes to LPS stimulation were then measured. The results showed that the quantities of TNF-α and IL1β released after LPS treatment were similar in both wild-type and CaV3.1−/− leucocytes (see Supplementary material online, Figure S2). These results suggest that the observed reduction of neointimal formation in CaV3.1−/− mice was not caused by reduced leucocyte recruitment or altered leucocyte function.

We then used BrdU labelling to investigate the correlation between defective smooth muscle proliferation and reduced neointimal formation in CaV3.1−/− mice. The results showed that in wild-type mice, the majority of the BrdU-positive cells were localized in the neointima of the injured arteries (Figure 2A). In contrast, in CaV3.1−/− mice, the quantity of BrdU-positive cells was significantly reduced, and they were mainly localized in the media. Furthermore, the neointimal cell proliferation index was significantly lower in CaV3.1−/− injured arteries than in wild-type controls (Figure 2B). These results suggest that the reduced neointimal formation in CaV3.1−/− mice after artery injury may be caused by defects in smooth muscle proliferation.

3.2 Upregulation of CaV3.1 mRNA and protein during neointimal formation in the injured vessels
Although CaV3.1 is expressed in the pulmonary artery and is involved in PASMC proliferation in humans and rats, CaV3.1−/− mice do not display any notable vascular abnormalities or abnormal blood pressure. Since neointimal formation was reduced in the injured arteries of the CaV3.1−/− mice, we hypothesized that CaV3.1 may be involved in the pathogenesis of neointimal formation. To verify this hypothesis, we examined the expression of CaV3.1 in neointima after artery injury and found that CaV3.1 mRNA was significantly increased by 2- and 5.7-fold at 1 and 2 weeks after wire injury, respectively (Figure 3A). CaV3.1 mRNA was upregulated 1 week after wire injury when there was no detectable neointimal formation (Figure 3A, left panel inset), suggesting that CaV3.1 is crucial for neointimal formation. Immunofluorescent staining with anti-CaV3.1 antibodies also showed a dramatic increase of CaV3.1 in the neointima of the injured arteries 2 weeks after wire injury in wild-type mice, whereas there was no detectable signal in the wild-type control or CaV3.1−/− arteries (Figure 3B).
Cav3.1 was required for wire injury-induced neointimal formation. (A) Elastic and smooth muscle α-actin staining of control and injured carotid arteries from wild-type (WT), Cav3.1\(^{-/-}\), and Cav3.2\(^{-/-}\) mice subjected to wire injury for 2 weeks. (B) Quantitation of intima/media (I/M) ratio of carotid arteries that were injured for 2 weeks. The I/M ratio (0.092 ± 0.022) of Cav3.1\(^{-/-}\) mice was significantly lower compared with those of wild-type and Cav3.2\(^{-/-}\) mice (0.659 ± 0.172 and 0.471 ± 0.146, respectively; *P = 0.021, n = 7 for each group). (C) H & E and von Willebrand factor immunohistochemical (vWF IHC) staining of control and injured carotid arteries from wild-type and Cav3.1\(^{-/-}\) mice subjected to wire injury for 24 h. (D) Similar vascular injury degree in wild-type and Cav3.1\(^{-/-}\) mice (0.914 ± 0.030 and 0.906 ± 0.020, respectively, P = 0.817, n = 6 for each group). (E) Sections of control and injured carotid arteries stained for pan-leucocytes (anti-CD45) from wild-type and Cav3.1\(^{-/-}\) mice subjected to wire injury for 1 week. (F) Quantitation of CD45 positive cells from carotid arteries of wild-type and Cav3.1\(^{-/-}\) mice (5.0 ± 1.4 and 4.0 ± 1.3, respectively, P = 0.616, n = 7 for each group) subjected to wire injury for 1 week.
Figure 2 Reduced neointimal VSMCs proliferation in Ca_{3.1}^{-/-} mice. (A) BrdU staining of control and injured carotid arteries from wild-type and Ca_{3.1}^{-/-} mice subjected to wire injury for 2 weeks. (B) Proliferation index of injured carotid arteries from wild-type and Ca_{3.1}^{-/-} mice (0.261 ± 0.063 and 0.013 ± 0.006, respectively) subjected to wire injury for 2 weeks. Proliferation index = BrdU positive cell number/total cell number in neointima and media. \( n = 7 \) for each group. **\( P = 0.008 \), compared with the wild-type group.

Figure 3 Upregulation of Ca_{3.1} mRNA and protein during neointimal formation. (A) RT–qPCR analysis of the expression of Ca_{3.1} in carotid arteries after 1 week and 2 weeks of wire injury in wild-type mice. \( n = 3 \) for each group. Each set of experiments contained four carotid arteries. *\( P = 0.028 \), **\( P = 0.001 \) compared with the control group. Inset shows elastic staining of injured carotid arteries from wild-type mice subjected to wire injury. (B) Sections of carotid arteries stained for Ca_{3.1} and smooth muscle \( \alpha \)-actin from injured wild-type, control wild-type, and Ca_{3.1}^{-/-} mice.
3.3 Deficiency of Ca$_{\text{a}}$3.1 reduced VSMC proliferation

To confirm that a loss of Ca$_{\text{a}}$3.1 impairs VSMC proliferation, we compared the growth curve of isolated aorta VSMCs from wild-type and Ca$_{\text{a}}$3.1$^{-/-}$ mice (Figure 4A). Our results showed that the proliferation rate of Ca$_{\text{a}}$3.1$^{-/-}$ VSMCs was significantly lower compared with that of wild-type VSMCs. Due to the low amount of isolated mouse VSMCs, we used rat aorta VSMCs as our in vitro cell model to study the signalling pathway triggered by the Ca$^{2+}$ influx via Ca$_{\text{a}}$3.1. Using an RNAi technique, we first showed that shCav3.1 repressed the level of Ca$_{\text{a}}$3.1 mRNA to 35% compared with the untreated controls (Figure 4B), whereas shLuc had no effect on the Ca$_{\text{a}}$3.1 mRNA level. Similar to the proliferation rate in Ca$_{\text{a}}$3.1$^{-/-}$ VSMCs, the proliferation rate in Ca$_{\text{a}}$3.1 knock down rat VSMCs was also reduced (Figure 4C).

Previous studies have shown that T-channels are predominantly expressed in the G$_1$ and S phases but not in the G$_0$ phase, so a reduced level of Ca$_{\text{a}}$3.1 may affect cell-cycle progression. To validate this hypothesis, we detected the levels of cyclin E during cell proliferation in the shLuc- and shCav3.1-treated rat VSMCs. Cyclin E, an activator of Cdk2, is tightly regulated during cell-cycle progression, and its expression level peaks at the G$_1$/S phase boundary followed by a rapid decline during the S phase. From PI flow cytometry results, we found that wild-type VSMCs progressed into the S phase at about 24 h after 10% FBS stimulation, whereas shCav3.1-treated VSMCs displayed a delayed S-phase progression (see Supplementary material online, Figure S3). We next established a time course experiment to detect the dynamics of cyclin E levels from 16 to 28 h after serum stimulation in shLuc- and shCav3.1-treated VSMCs. Our results showed that in shLuc-treated VSMCs, the level of cyclin E peaked at 20 h and declined at 24 h. In contrast, in shCav3.1-treated VSMCs, the cyclin E level remained relatively constant throughout the time course (Figure 4D). We next detected the ratio of cyclin E levels between 20 and 24 h (20/24 h) in shCav3.1-treated VSMCs. The ratio of the cyclin E levels between 20 and 24 h in the shLuc-treated VSMCs was significantly higher than that of the shCav3.1-treated VSMCs (Figure 4E). These results suggest that a deficiency or knock down of Ca$_{\text{a}}$3.1 may reduce VSMC proliferation by delaying the G$_1$-to-S transition, and that Ca$_{\text{a}}$3.1 plays a role in cyclin E signalling.

3.4 Calmodulin agonist CALP1 increased cell proliferation in Ca$_{\text{a}}$3.1 knockdown VSMCs

It has been shown that CaM-dependent cyclin E/Cdk2 activity is required for the Ca$^{2+}$-sensitive G$_1$/S phase transition in VSMCs. However, the source of Ca$^{2+}$ which activates this signalling pathway is unclear. We hypothesized that the Ca$^{2+}$ influx via Ca$_{\text{a}}$3.1 activates the Ca$^{2+}$/CaM pathway to promote the G$_1$-to-S phase transition in VSMCs. To verify our hypothesis, we
treated VSMCs with CALP1, a cell-permeable CaM agonist that binds to the EF hands of CaM.38 We found that CALP1 (10 μmol/L) enhanced cell proliferation in the shCa v3.1-treated VSMCs (Figure 5), but it had no effect on the cell proliferation rate of wild-type (A) and shLuc-treated (B) VSMCs. (C) CALP1 (10 μmol/L) increased the cell proliferation rate of shCa v3.1-treated VSMCs. The cell proliferation rate was measured using a CyQUANT cell proliferation assay. *P < 0.001, compared with the shCa v3.1-treated group.

### 3.5 NNC55-0396 dihydrochloride, a selective T-channel blocker, inhibited neointimal formation

Having demonstrated that Cav3.1 was involved in the VSMC proliferation during neointimal formation after artery injury, we explored the possibility of using T-channel blockers as a therapeutic tool to
prevent neointimal formation. We used a T-channel selective blocker, NNC55-0396, to validate our hypothesis. We first determined the effect of NNC55-0369 on VSMC proliferation, and the results showed that NNC55-0369 was able to decrease the proliferation rate of rat VSMCs (Figure 6A). We also found that NNC55-0369 was able to decrease the platelet-derived growth factor induced \([\text{Ca}^{2+}]_i\), elevation in VMSCs (see Supplementary material online, Figure S4). We then tested the effect of NNC55-0396 in vivo. Neointimal formation was significantly reduced in NNC55-0396-treated mice compared with the saline controls (Figure 6B). Moreover, the I/M ratio was significantly decreased in the NNC55-0396-treated group compared with the saline-treated controls (Figure 6C). These results support the idea that a specific T-channel blocker could serve as an effective agent to prevent neointimal formation, thus reducing the severity of restenosis after artery intervention.

4. Discussion

In this study, we demonstrated that wire injury-induced neointimal formation was abolished in mice lacking Ca\(_{\text{v}3.1}\). The involvement of T-channels in neointimal formation is unique to Ca\(_{\text{v}3.1}\), because neointimal formation also occurs in Ca\(_{\text{v}3.2}^{\text{+/−}}\) mice. We further demonstrated that Ca\(_{\text{v}3.1}\) was upregulated in the VSMCs during neointimal formation in injured carotid arteries. RNAi and CaM agonist studies showed that Ca\(_{\text{v}3.1}\) was required for VSMC proliferation in the G\(_1\)/S phase transition, possibly via a CaM-dependent signalling pathway. Furthermore, we also showed that a T-channel blocker, NNC55-0396, was able to reduce the neointimal formation after vascular injury in wild-type mice. In summary, our results demonstrated that Ca\(_{\text{v}3.1}\) is required for VSMC proliferation during neointimal formation, and that Ca\(_{\text{v}3.1}\) may be a potential therapeutic target to prevent vascular restenosis.

Although both Ca\(_{\text{v}3.1}\) and Ca\(_{\text{v}3.2}\) are expressed in VSMCs,\(^{14,15}\) their roles in the VSMCs are not completely understood. Ca\(_{\text{v}3.1}\) is expressed in smooth muscles of rat mesenteric arterioles, and mediates Ca\(_{\text{v}3.2}\) entry during conducted vasoconstriction,\(^{39}\) whereas Ca\(_{\text{v}3.2}\) is involved in coronary artery relaxation and conducted vasodilator response in cremaster arterioles.\(^{27,40}\) Therefore, Ca\(_{\text{v}3.1}\) and Ca\(_{\text{v}3.2}\) seem to play different roles in the conducted vascular responses. Other studies have shown that Ca\(_{\text{v}3.1}\) is involved in VSMC proliferation in human pulmonary arteries,\(^{16}\) and that it is also involved in the closure of neonatal rat ductus arteriosus induced by oxygenation.\(^{17}\) Our study is the first to show that Ca\(_{\text{v}3.1}\), but not Ca\(_{\text{v}3.2}\), is crucial for neointimal formation in injured arteries. In addition, Ca\(_{\text{v}3.1}^{\text{+/−}}\) mice display bradycardia without any notable abnormalities in vascular morphology or abnormal blood pressure.\(^{32}\) This indicates that Ca\(_{\text{v}3.1}\) is probably not involved in blood vessel formation during development. We also found that Ca\(_{\text{v}3.1}\) was only detected in the VSMCs of injured arteries, but not in the VSMCs of uninjured arteries. Furthermore, the upregulation of Ca\(_{\text{v}3.1}\) mRNA occurred before neointimal formation in the injured arteries. These results suggest that Ca\(_{\text{v}3.1}\) is required for the contractile to proliferative phenotype transition of VSMCs in injured arteries.

Other ion channels such as \(K^{+}\) channels are also known to contribute to the proliferation of VSMCs. Down-regulation of large conductance \(K_{\text{v}}^{\text{Ca}^{2+}}\)-activated \(K^{+}\) channel \(K_{\text{v}}^{\text{Ca}^{2+}}\) have been described in VSMCs during the transition from contractile to proliferative phenotype.\(^{41}\) Up-regulation of \(K_{\text{v}}^{3.4}\) and \(K_{\text{v}}^{1.3}\) has also been observed in proliferating human and rat smooth muscle cells, respectively.\(^{42,43}\) Selective blockade of \(K_{\text{v}}^{3.1}\) inhibited smooth muscle cell proliferation and reduced neointimal formation in a rat model.\(^{44}\) Opening of these \(K^{+}\) channels can lead to membrane hyperpolarization, and hyperpolarized resting membrane potentials would favour the opening of Ca\(_{\text{v}3.1}\) T-channels in proliferating VSMCs.

T-channels have been implicated in the regulation of cell-cycle progression and cell proliferation.\(^{45}\) In rat aorta VSMCs, T-channels are mainly expressed in the G\(_1\) and S phases but not in the G\(_0\) phase.\(^{13}\) Another study also showed that T-channels are predominantly expressed in the S phase in rat neonatal cardiac myocytes.\(^{46}\) Transient elevation in \([\text{Ca}^{2+}]_i\), is associated with cell-cycle progression, especially in the G\(_1\) phase and mitosis.\(^{47,48}\) It has been reported that a coordinated elevation in \([\text{Ca}^{2+}]_i\), is required for the G\(_1\)/S-phase transition in rat VSMCs.\(^{9}\) In eukaryotic cells, one of the major \(Ca^{2+}\)/signal transducers for cell-cycle progression is CaM and the \(Ca^{2+}/CaM\) signalling pathway.\(^{39}\) Studies have revealed that the kinase activity of cyclin E/Cdk2, but not other G\(_1\)/S-associated cell-cycle complexes, is sensitive to physiological changes in \([\text{Ca}^{2+}]_i\), in VSMCs. Furthermore, the CaM-binding site of cyclin E has been shown to mediate the \(Ca^{2+}\)/sensitive G\(_1\)/S transitions in VSMCs in vitro.\(^{36}\) Husain and colleagues further demonstrated the importance of CaM–cyclin E interaction in VSMC proliferation and neointimal formation in vivo. Their study found decreased VSMC proliferation and neointimal formation after the disruption of CaM–cyclin E interaction.\(^{37}\) However, the source of calcium stimuli for this activation pathway remains unclear. Recently, Pluteanu and Cribs showed that \(Ca^{2+}\)/ influx via Ca\(_{\text{v}3.1}\) is involved in IGF-1 stimulated PASMС proliferation.\(^{18}\) They showed that knockdown of Ca\(_{\text{v}3.1}\) inhibited both IGF-1 stimulated and basal cyclin D mRNA expression levels. We hypothesized that the \(Ca^{2+}\)/ influx via Ca\(_{\text{v}3.1}\) activates the Ca\(_{\text{v}3.1}\)–calmodulin–cyclin E pathway, and hence promotes the G\(_1\)/S-phase transition of VSMCs. Our results from PI flow cytometry showed that 24 h after FBS stimulation, rat aorta VSMCs progressed into the S phase, whereas more time was required for shCa\(_{\text{v}3.1}\)-treated VSMCs to progress. We also demonstrated that the tightly regulated cyclin E expression pattern was disrupted in the Ca\(_{\text{v}3.1}\) knockdown cells. As expected, CaM agonist CALP1 was able to increase the proliferation of Ca\(_{\text{v}3.1}\) knockdown VSMCs, but it had no effect on the proliferation of wild-type and shLuc VSMCs. These results support our hypothesis that Ca\(_{\text{v}3.1}\) Ca\(_{\text{v}3.1}\) \(Ca^{2+}\)/calmodulin–cyclin E pathway to promote the G\(_1\)/S-phase transition of VSMCs. However, we observed only partially rescued cell proliferation, and we cannot rule out the possibility that Ca\(_{\text{v}3.1}\) \(Ca^{2+}\)/ influx may activate other pathways such as increasing transcription of cyclin D as observed previously.\(^{18}\)

Previous studies have shown that CCBs are probably effective in reducing restenosis in clinical trials.\(^{19–21}\) Currently, most of the CCBs used are L-channel blockers, however, downregulation of L-channels is known to occur in proliferating VSMCs.\(^{50,51}\) It is known that many CCBs not only block L-channels, but also block T-channels to a different extent.\(^{23,24}\) Therefore, it is likely that some effects of CCBs in reducing restenosis result from the blocking of T-channels. In this study, we used NNC55-0396, a CCB that irreversibly blocks calcium T-channels,\(^{52,53}\) to examine the reduction in neointimal formation in wild-type mice after vascular injury in vivo. Our results showed that infusion of NNC55-0396 for 7 days was effective in preventing neointimal formation in wild-type injured arteries. These results are consistent with our finding that Ca\(_{\text{v}3.1}\) mRNA was upregulated 7
days after artery injury when there was no obvious neointimal formation. Our results provide a new insight into the regulatory mechanism of Ca3.1 in cell proliferation in VSMCs, and also demonstrate that Ca3.1-specific CCBs may be more beneficial than other inhibitors to prevent restenosis after PCI.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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