Role of Girdin in intimal hyperplasia in vein grafts and efficacy of atelocollagen-mediated application of siRNA for vein graft failure

Hiroki Miyachi, MD,[a,b] Shinji Mii, MD, PhD,[a,c] Atsushi Enomoto, MD, PhD,[a] Yoshiki Murakumo, MD, PhD,[a,d] Takuya Kato, PhD,[a] Naoya Asai, MD, PhD,[c] Kimihiro Komori, MD, PhD,[b] Masahide Takahashi, MD, PhD[a,c,*]

[a]Department of Pathology, Nagoya University Graduate School of Medicine, Nagoya, Japan.

[b]Division of Vascular Surgery, Department of Surgery, Nagoya University Graduate School of Medicine, Nagoya, Japan.

[c]Division of Molecular Pathology, Center for Neurological Disease and Cancer, Nagoya University Graduate School of Medicine, Nagoya, Japan.

[d]Department of Pathology, Kitasato University School of Medicine, Sagamihara, Japan.

[*]**Correspondence:** M Takahashi, Department of Pathology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan. Tel: +81 52 744 2093; Fax: +81 52 744 2098; E-mail: mtakaha@med.nagoya-u.ac.jp

Sources of Funding

This work was supported by Grants-in-Aid for Global Center of Excellence (GCOE) research, Scientific Research on Innovative Area, Scientific Research A (M.T.) and Scientific Research B (K.K.) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

Disclosures

The authors declare no conflict of interest.

Abstract

Introduction: Intimal hyperplasia is a major obstacle to patency in grafted veins. Although migration and proliferation of vascular smooth muscle cells (SMCs) pivotally affect the vascular remodeling process, no therapy has been established to prevent intimal hyperplasia of vein grafts. We previously reported that the actin-binding protein Girdin crucially affects arterial remodeling. In this study, we investigated the role of Girdin in venous SMCs, and evaluated a therapeutic strategy for vein graft failure *in vivo* using small interfering RNA (siRNA) that targets *Girdin*.

Methods: We investigated the relationship between Girdin expression and intimal hyperplasia using a rabbit vein graft model. Vein grafts under low flow conditions were performed in Japanese white rabbits. For *in vitro* analyses, we isolated primary venous SMCs from vein graft neointima. siRNA that targets *Girdin* was mixed with atelocollagen (which stabilizes and releases nucleic acid reagents slowly) and applied perivascularly to vein grafts at operation. Intimal hyperplasia was evaluated 4 weeks later.

Results: In the rabbit model, increased Girdin expression was seen in the neointima after grafting operation. Using primary venous SMCs, we showed that Girdin is required for rearrangement of the actin cytoskeleton in venous SMCs, and that siRNA-mediated *Girdin* knockdown significantly reduced venous SMC migration and proliferation. *Girdin*

knockdown via perivascular application of siRNA using atelocollagen markedly reduced intimal thickening after grafting operation.

Conclusions: Depletion of Girdin attenuated venous SMCs migration and proliferation *in vitro* and intimal hyperplasia in vein grafts *in vivo*. Our findings suggest that Girdin affects migration and proliferation of vascular SMCs in vein grafts and that controlled release of *Girdin* siRNA using atelocollagen could be a novel therapeutic strategy for vein graft failure.

Clinical relevance: Intimal hyperplasia is a major obstacle to patency after vein grafting. Although migration and proliferation of vascular smooth muscle cells pivotally affect the vascular remodeling process, no therapy has been established to prevent intimal hyperplasia of vein grafts. Here, we report that depletion of the actin-binding protein Girdin attenuated migration and proliferation of venous smooth muscle cells *in vitro* and intimal hyperplasia in vein grafts *in vivo*. Our findings indicate that perivascular application of *Girdin* siRNA using atelocollagen could be a novel therapeutic strategy for vein graft failure.

Introduction

Cardiovascular disease (CVD) is a major cause of death and disability in developed countries. More than 2200 Americans die of CVD each day, an average of 1 death every 39 seconds.[1] An autologous vein is the most commonly used conduit for coronary and peripheral artery bypass grafting in treating ischemia resulting from occlusive vascular disease. However, long-term success of this operation is limited by vein graft occlusion caused by intimal hyperplasia and superimposed atherosclerosis, with worse patency for poor run-off vessels in which hemodynamic factors, such as low flow velocity and low shear stress, cause intimal thickening.[2,3] Increased proliferation of terminally differentiated vascular smooth muscle cells (SMCs) contributes significantly to lesional neointima formation.[4] Neointimal SMCs are controversially reported to originate from local vessel walls, circulating progenitor cells, or from bone marrow.[5–9] Despite many clinical trials, few models reflect the contribution of various cell types to neointimal development; nor has a satisfactory therapeutic strategy been established.

The phosphatidylinositol 3-kinase (PI3K)/Akt system regulates multiple cellular processes through phosphorylation of downstream substrates. The PI3K/Akt pathway and its downstream components are also pivotal in vascular remodeling.[10] Girdin (girders of actin filaments), also known as Gα-interacting vesicle-associated protein (GIV), is a novel actin-binding Akt substrate.[11–14] Girdin is expressed at high levels in cell types that require

remodeling of actin filaments, cell migration and proliferation, such as smooth muscle cells, neuroblasts, and cancer cells.[11,15–19]

We reported that Girdin affects migration and proliferation of arterial SMCs, and regulates neointima formation after arterial injury via Akt-mediated phosphorylation .[18] However, little information is available about its functions in venous SMCs, especially in vein grafts. Here, we studied Girdin's roles in migration and proliferation of venous SMCs *in vitro*; and found a combination of siRNA that targets *Girdin* and atelocollagen (which allows its controlled release), to suppress significantly intimal hyperplasia in vein grafts *in vivo*. These findings provide a novel strategy to prevent intimal hyperplasia.

Material and methods

Antibodies

Rabbit anti-Girdin polyclonal antibody was developed in-house against the 19 carboxyl-terminal amino acids of Girdin, and affinity-purified with the immunizing peptide.[11] Other primary antibodies used in this study include commercial sheep polyclonal anti-Girdin (R&D Biosystems, Minneapolis, MN), mouse monoclonal anti-α-smooth muscle actin (α-SMA) and anti-Ki-67 antibodies (Dako, Glostrup, Denmark), mouse monoclonal anti-β-actin antibody (Sigma, St Louis, MO) and rat monoclonal anti-CD31 antibody (Dianova, Hamburg, Germany).

Vein graft model

Japanese white rabbits (2.5–3.0 kg) were anesthetized by intramuscular administration of ketamine (35 mg/kg) and Xylazine (10 mg/kg). The no-touch technique was used to dissect a segment of right external jugular vein (20 mm length, 3 mm width) through a midline vertical neck incision; all branches were carefully ligated with 8-0 polypropylene sutures and divided. Animals were systemically heparinized (200 U/kg). The ipsilateral common carotid artery was clamped distally and proximally, and a graft was anastomosed in end-to-end fashion into the divided artery with interrupted 8-0 polypropylene sutures. Before wound closure, grafts were subjected to low flow conditions as described previously (poor run-off model).[20,21] The poor run-off model mimics clinical conditions of vein grafts performed for ischemic extremities with poor run-off. For poor run-off models, three of four distal vessel branches were ligated after external jugular vein–carotid artery interposition (Figure 1A).[2,3] On the other hand, the distal vessels remained patent in the normal run-off model. These animal experiments were performed in compliance with the guidelines of Nagoya University Graduate School of Medicine and approved by the Animal Care and Use Committee (Permit Number: 24084).

Primary culture of venous SMCs

The experiments were performed with primary cultures of venous SMCs from rabbit vein grafts 4 weeks after bypass grafting. The entire length of the vein grafts were

immediately excised under sterile conditions. The vein grafts were rinsed with Hanks' solution and opened lengthways. The neointima was dissected from underlying media. The separated sheets of neointima were cut into small pieces of about 1 mm^2,washed in Hanks' solution, and incubated in Hanks' solution containing collagenase I (1 g/L, Funakoshi, Tokyo, Japan) and elastase III (0.5 g/L, Sigma) for 90 min at 37°C. Thereafter the cells were dispersed; the cell suspension was centrifuged at 120 × *g* for 4 min, resuspended and grown in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS, 1% streptomycin/penicillin, and 2 mM L-Glutamine. The identity of SMCs was confirmed by immunostaining with anti-α-SMA antibody. Venous SMCs at passages 1–3 were used in the experiments.

RNA interference

Small interfering RNA-mediated knockdown of *Girdin* was performed using "siRNA [B]" *in vitro* as described previously.[11,18] Additionally, we made two more siRNAs for rabbit Girdin, as follows (sense sequence only: 5′-GGACCAACCUGGAUGAAUATT-3′ (nucleotides 98–116, si-rb1) and 5′-GGCAGAACAUCCACUAGCATT-3′ (nucleotides 4768–4786, si-rb2). The 21-nucleotide synthetic duplexes with two 3′-end overhang dT were prepared by Qiagen (Valencia, CA). Venous SMCs were transfected with one of the siRNAs (50 nM) or a 21-nucleotide irrelevant RNA (Qiagen) as a control, using Lipofectamine

RNAiMAX (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. siRNAs were successfully introduced into almost 100% of SMCs using Lipofectamine RNAiMAX.

For the *in vivo* study, we used stabilized siRNAs, Dharmacon siSTABLE (Thermo Scientific, Lafayette, CO). We used atelocollagen-based *in vivo* siRNA/miRNA transfection reagent AteloGene Local Use (Koken Co, Ltd, Tokyo, Japan). AteloGene is a highly purified type I collagen derived from calf dermis by pepsin treatment. Before the operation, siRNA–AteloGene complexes were prepared as follows: 150 μL of AteloGene and 150 μL of a stabilized siRNA solution (20 μM) were mixed, maintained at 4°C, and then used to coat the external surface of the vein graft. This complex formed a gel after appropriate heat treatment with a hair dryer for < 10 s, which did not dry or injure the vein grafts.[20] This siRNA-containing gel remained around the graft for at least 1 week.

Western blot analysis

Frozen rabbit vein grafts were homogenized in RIPA buffer containing 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), and 0.1% sodium deoxycholate and rotated at 4 rpm for 2 h at 4°C. After measuring protein concentrations, lysates were boiled at 100°C for 2 min in the presence of 2% β-mercaptoethanol. The lysates, containing 30 μg of proteins, were subjected to SDS-polyacrylamide gel electrophoresis. Western blotting was performed using standard protocols detailed in Supplemental Methods.

Histopathology

Intact veins and vein grafts were fixed at 100 mmHg infusion pressure for 20 min with 10% neutral-buffered formalin and immersed for 40 h in 10% neutral-buffered formalin; 3 evenly spaced segments (4–5 mm apart) of the middle portion of each graft were then embedded in paraffin. Serial sections were stained with hematoxylin and eosin or elastica van Gieson stain. Intimal and medial cross-sectional areas were measured, and the neointimal thickness was measured at eight randomly selected points for each section using MetaMorph software (Universal Imaging, Ypsilanti, MI).

Immunohistochemistry

Sections were heated in Target Retrieval Solution (pH 9.0; Dako) for antigen retrieval, blocked with Protein Block Serum-Free Ready-to-Use (Dako), incubated with primary antibodies, washed in PBS and incubated with the secondary antibody. Reaction products were visualized with diaminobenzidine (Dako); nuclear counterstaining used hematoxylin.

Immunofluorescence staining

Immunofluorescence staining was performed as previously described[11]; protocols are detailed in Supplemental Methods.

Quantification of lamellipodia

We investigated the increased number of cells with extended lamellipodia, that is the difference in number of cells with extended lamellipodia between before and after PDGF stimulation. Note that cells with lamellipodia at more than one third (> 120°) of the cell periphery were counted as cells with extended lamellipodia.

Cell migration assay and WST-1 assay

Directional cell migration of venous SMCs was stimulated in a monolayer using an *in vitro* scratch wound assay as previously described[15]; protocols are detailed in Supplemental Methods.

Mitogenic activity was measured using the WST-1 assay (Cell Proliferation Reagent WST-1; Roche Applied Science, Penzberg, Germany); protocols are detailed in Supplemental Methods.

Detection of cell proliferation and apoptosis in vivo

Ki-67+ or TUNEL+ nuclei were evaluated as previously described.[18,20] To detect proliferation, sections of rabbit vein grafts were blocked and subsequently incubated with a mouse monoclonal anti-Ki-67 antibody overnight at 4°C. Thereafter, sections were incubated with EnVision+ System- HRP Labelled Polymer Anti-mouse (Dako). To detect apoptosis, we used Apoptosis *in situ* Detection Kit (Wako, Osaka, Japan) according to the manufacturer's

protocol. In each experiment, hematoxylin was used for nuclear counterstaining. Numbers of Ki-67+ and TUNEL+ cells were calculated as Ki-67 or TUNEL indices (Ki-67+ or TUNEL+ cells/total cells × 100), respectively.

Statistical analysis

Values are expressed as means ± SEM. Significance ($P < .05$) was determined using Student's *t*-test or Bonferroni post hoc analysis.

Results

Increased expression of Girdin in neointima of vein grafts

To examine the effects of Girdin in vein grafts, Girdin expression in cross sections prepared from rabbit vein grafts with poor run-off was assessed immunohistochemically. Girdin expression was detected in the media of the intact vein which was α-SMA+ (Figure 1B, Supplemental Figure 1). As the neointima thickened, enhanced Girdin expression was observed in the neointima rather than the media (Figure 1B). Girdin expression in neointima peaked 14 days after bypass grafting, and gradually decreased thereafter.

Girdin expression in whole intact veins and vein grafts with poor run-off was studied using Western blot analyses (Figure 1C). Only weak Girdin expression was detected in intact veins. Expression levels increased after vein grafting and peaked at day 14 after bypass grafting, which was consistent with immunohistochemical analysis. To confirm Girdin

localization, 14-day-old vein grafts were subjected to immunofluorescence staining using anti-Girdin antibody and anti-α-SMA antibody (Figure 1D). Girdin localizes to α-SMA+ cells in media and neointima. Cells that were both Girdin+ and α-SMA+ existed mainly in the vein graft neointima at 14 days after bypass grafting. This finding suggests that Girdin is upregulated in developing neointima.

Girdin is upregulated in the vein grafts with poor run-off

To determine whether Girdin affects intimal hyperplasia in vein grafts, we compared Girdin expression in vein grafts with poor and normal run-off. The neointima clearly thickened in vein grafts with poor run-off compared with normal run-off models (Figure 2A)—consistent with previously reported data.[3] All vein grafts of both models were patent when they were excised. Western blot analyses showed Girdin expression to markedly increase during neointima formation at 7 and 14 days after bypass grafting in poor run-off models compared with normal run-off models (Figure 2B).

Rearrangement of actin cytoskeleton of venous SMCs requires Girdin

We examined Girdin localization using primary venous SMCs isolated from neointima of vein grafts. Immunocytochemistry using anti-Girdin and anti-β-actin antibodies showed Girdin localized on actin stress fibers in venous SMCs (Figure 3A), as previously observed in other cell types.[15,18] To investigate Girdin function in venous SMCs, we observed the effect of RNA-mediated interference on actin cytoskeletons in these cells. We introduced three

siRNAs targeting *Girdin* (siRNA [B], si-rb1, si-rb2) and an irrelevant siRNA (control siRNA) into venous SMCs. Although serum-starved controls and Girdin-depleted venous SMCs showed no apparent morphological differences, formation of thick stress fibers significantly decreased in Girdin-depleted cells stimulated with serum compared with control cells (Figure 3B). Moreover, after stimulation with PDGF-BB, the leading-edge extension of lamellipodia was significantly attenuated, and only small membrane protrusions could be seen in the Girdin-depleted cells (Figure 3C). The increased number of Girdin-depleted cells with extended (> 120°) lamellipodia after PDGF stimulation was significantly lower than that of control cells (14.49 ± 3.19 and 1.95 ± 1.57 per 100 cells, respectively; n = 3; $P < .05$; Figure 3D). These findings suggest that Girdin pivotally affects actin remodeling of lamellipodia in migrating venous SMCs.

Girdin depletion inhibits venous SMCs migration and proliferation in vitro

Reportedly, vascular SMCs migration and proliferation are key events in intimal hyperplasia of vein grafts.[4] We investigated whether Girdin depletion inhibits venous SMCs migration *in vitro* using wound healing assays (Figure 4A); siRNA-mediated *Girdin* knockdown resulted in significantly reduced migration distance by venous SMCs and fewer cells migrating into the wounded area, compared with those of venous SMCs transfected with control siRNA (n = 3; $P < .01$; Figure 4B and 4C). Next, we examined the effect of Girdin on venous SMC proliferation, using WST-1 assays. On day 4, proliferation of Girdin-depleted

cells was significantly suppressed compared with control cells (n = 5; $P < .01$; Figure 4D). These findings show that Girdin depletion inhibits both migration and proliferation of venous SMCs *in vitro*, suggesting that use of siRNA targeting *Girdin* could be a novel treatment for intimal hyperplasia of vein grafts

Girdin depletion inhibits intimal hyperplasia in vein grafts in vivo

Because the siRNAs targeting *Girdin* (siRNA [B], si-rb1, si-rb2) had similar biological effects *in vitro* (Figures 3 and 4, data not shown) and transfection with si-rb2 tended to more strongly suppress Girdin expression (Supplemental Fig. 2A), we adopted the sequence of si-rb2 for *in vivo* study. After initially confirming that stabilized siRNA (siSTABLE) retained inhibitory effects equal to those of unmodified siRNA (Supplemental Fig. 2B), we used siSTABLE for all animal experiments. Vein grafts were treated with *Girdin* siRNA as described in Material and methods. We applied *Girdin* siRNA mixed with atelocollagen perivascularly to vein grafts at operation (Supplemental Fig.3A). Western blot analysis confirmed that *Girdin* siRNA effectively suppressed Girdin expression in vein grafts *in vivo* at least for 14 days post-operation (Figure 5A); we therefore evaluated its potential to inhibit intimal hyperplasia. Four weeks post-operation, intimal thickness and the intima/media ratio of vein grafts treated with atelocollagen-mediated local application of *Girdin* siRNA were 0.09 ± 0.01 mm and 0.67 ± 0.08, respectively (n = 5), whereas those of grafts treated with atelocollagen containing irrelevant siRNA were 0.22 ± 0.05 mm and 1.76 ± 0.24, respectively

(n = 5; Figure 5B–D). Additionally, intimal thickness and the intima/media ratio did not significantly differ between vein grafts treated with atelocollagen containing irrelevant siRNA and non-treated grafts, indicating that controlled release of *Girdin* siRNA using atelocollagen reduced intimal hyperplasia of rabbit vein grafts significantly ($P < .05$), whereas atelocollagen alone had no effect. However, medial thickness did not differ among the three groups at 4 weeks post-operation (Supplemental Fig. 3B).

Increased endothelial coverage reportedly correlates with diminished intimal hyperplasia.[22] To further investigate the effects of *Girdin* knockdown in vein grafts, we evaluated re-endothelialization in vein grafts 7 days after bypass grafting. Endothelial coverage in vein grafts was assessed immunohistochemically using anti-CD31 antibody (Supplemental Fig. 4A). Quantification with image analysis showed no significant difference in endothelial coverage between *Girdin* siRNA-treated vein grafts and control siRNA-treated grafts (Supplemental Fig.4B). This suggests that the inhibitory effect of *Girdin* knockdown on intimal hyperplasia was unrelated to re-endothelialization.

Girdin is involved in cell proliferation, but not cell survival, in vein grafts

Cell migration, cell proliferation and apoptosis of vascular SMCs are major steps in intimal hyperplasia.[23,24] To examine cell proliferation and apoptosis in vein grafts, we performed Ki-67 immunostaining and TUNEL staining, respectively (Figure 6A and 6D). Vein grafts treated with *Girdin* siRNA showed significantly fewer Ki-67+ proliferating cells

from media to neointima at 7 days post-operation compared with vein grafts treated with control siRNA (Figure 6B). Media and neointima were difficult to distinguish at 7 days post-operation. Twenty-eight days post-operation, Ki-67+ cells significantly increased in neointima but not in media (Figure 6C). Conversely, cell survival in neither neointima nor media was affected significantly in vein grafts treated with *Girdin* siRNA at any point (Figure 6E, F). These results suggest that in vein grafts, Girdin affects cell proliferation, but not cell survival.

Discussion

As we previously reported that Girdin depletion reduces migration and proliferation of arterial SMCs,[18] we hypothesized that *Girdin* knockdown would retard intimal hyperplasia in vein grafts. Immunohistochemical analyses confirmed that Girdin localized to vascular SMCs in media and neointima of rabbit vein grafts. Girdin expression in neointima peaked at ~14 days after implantation. These changes in Girdin expression in vein grafts resembled those of vascular SMC proliferation in vein grafts.[25] In poor run-off models with abnormal blood flow conditions, as characterized by low flow and low shear stress variation mimicking clinical conditions of vein grafts performed for ischemic extremities, intimal graft thickening is more progressive than that in control models with normal run-off.[2,3] We showed that Girdin expression in vein grafts with poor run-off was greater than that in vein grafts with normal

run-off. These results suggest that Girdin contributes to vascular SMC proliferation in vein grafts and intimal hyperplasia after bypass grafting.

We isolated primary venous SMCs from enzyme-dispersed neointima of rabbit vein grafts 4 weeks post-operation. These primary cells, apparently originating from various cell types, exhibited increased migration and proliferation shortly after being isolated. In contrast, cells isolated from vein grafts 8 weeks post-operation in the same way did not migrate readily and rarely underwent mitosis in the first few days (data not shown). In this study, we isolated primary venous SMCs from neointima of vein grafts by the enzyme dispersion method, while isolation of venous SMCs by the explant method has recently been reported.[26] We suppose that cells prepared in this way reflect the form of vascular SMCs in vein graft more exactly than those isolated from explants of vena cava or saphenous veins, because cell types from neointimal SMCs are different from those of medial SMCs,[27] and the enzyme dispersion method is known to retain characteristic features of *in vivo* cells.[28] Our data showed that Girdin localized to venous SMCs especially in neointima of vein grafts 14 days after implantation, rather than those in media.

Plausibly, Girdin might be expressed in dedifferentiated vascular SMCs and affect phenotypic modulation in vascular SMCs because vein graft neointima have abundant dedifferentiated vascular SMCs.[21] To examine this hypothesis, we stimulated venous SMCs with PDGF-BB (a known inducer of phenotypic switch in vascular SMCs) containing 10%

FBS, which markedly enhanced Girdin expression (Supplemental Fig. 5A) and led to morphological change (Supplemental Fig. 5B). Although our data suggest that Girdin is up-regulated in dedifferentiated vascular SMCs, further studies are needed to understand the role of Girdin in vascular SMC phenotypic modulation.

Despite intense research, the optimal vector for gene delivery to vasculature remains somewhat elusive. Such a vector should efficiently transduce target vascular cells with minimal transduction of non-target cells, have low toxicity and immunogenicity, and be sufficiently stable to allow longevity of transgene expression, leading to an adequate clinical response.[29] Although the most commonly used vector is an adenovirus, viral vectors pose three main limitations in human vascular gene therapy: common preexisting immunity to adenovirus; profound immune response generated to adenoviral-transduced cells; and direct tissue toxicity.[30] Many trials, including genetic interventions to prevent vein graft failure, take advantage of the fact that veins to be grafted can be manipulated *ex vivo*. Antisense or decoy oligodeoxynucleotides (ODNs) have been transfected into graft vein walls via liposomes or high pressure.[31–35] Newer methods, such as ultrasound or gene guns, deliver desired genes via non-viral vectors; for example, plasmids through cellular walls[36]; however, this strategy allows a drug to be administered only once, and most of the applied drug remains at the application site relatively briefly. As neointimal SMCs have three potential sources—vein

grafts, arterial walls, and circulating blood—such single treatments for vein grafts are probably insufficient.[7]

Here, we used atelocollagen to deliver siRNA. Atelocollagen is a liquid at 4°C and becomes solid at 37°C. It has been used as a delivery system for expression plasmids, antisense ODNs and siRNAs, as it stabilizes and slowly releases nucleic acid reagents.[37–39] Thus siRNA is retained at the application site, allowing long-lasting target gene suppression in vascular SMCs without regard to cell origin. Also, siRNA was retained at the application site 7 days post-operation, as found in a study using fluorescence-labelled siRNA.[20]

We confirmed that Girdin expression was suppressed for at least 14 days using atelocollagen-mediated siRNA targeting *Girdin*. We also showed that Girdin expression peaked around 14 days after bypass grafting and treatment with atelocollagen-mediated siRNA targeting *Girdin* significantly inhibited intimal hyperplasia in vein grafts 28 days post-operation. Conversely, Girdin depletion did not affect medial thickness 28 days post-operation (Supplemental Fig. 3B), although neointimal SMCs may be derived from media without enhanced proliferation of medial SMCs (Figure 6C). The mechanism by which *Girdin* siRNA inhibits intimal hyperplasia remains unclear. Notably, Girdin depletion did not affect re-endothelialization in vein grafts, which is an important response consideration in bypass grafting.[22,40] Our study indicates that suppressing Girdin in vein grafts could be a useful strategy for preventing vascular stenosis.

In conclusion, Girdin is pivotal to migration and proliferation of venous SMCs isolated from neointima of rabbit vein grafts. We confirmed that the combination of atelocollagen and siRNA to allow controlled siRNA release could be a novel therapeutic strategy for vein graft failure, and that Girdin is a potential molecular target for prevention of intimal hyperplasia in vein grafts *in vivo*.

Acknowledgments

We thank Mr. Koichi Imaizumi, Mrs. Kaori Ushida, Mr. Kozo Uchiyama and Mrs. Akiko Itoh for technical assistance, and gratefully acknowledge Dr. Kenji Kadomatsu and Dr. Yoshifumi Takei for their information about atelocollagen.

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Figure legends

Figure 1. Girdin localizes to vascular SMCs in tunica media and neointima of vein grafts. **A**, Poor run-off vein graft model with distal branches ligation is depicted. The inferior-most branch of the external carotid artery is the only outflow for the low flow graft. CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; VG, vein graft. **B**, Rabbit vein grafts at the indicated days post-operation were subjected to immunohistochemical analysis using anti-Girdin antibody. Note that these veins and vein grafts are dilated with infusion pressure during fixation and vascular walls look thinner than under normal conditions. Bar: 100 μm. **C**, Western blot analyses show Girdin expression in rabbit whole-vein grafts. The open bar graph shows protein levels of Girdin (relative to β-actin) determined by densitometry in whole-vein grafts harvested at indicated days post-operation. Girdin level in the intact vein was defined as 1 (error bars: SEM; n = 3 for each group; **P* < .05). **D**, Vein grafts at 14 days post-operation were subjected to immunofluorescence staining using sheep IgG as a negative control or anti-Girdin antibody (red), and anti-α-SMA antibody (green). Cell nuclei were labeled with DAPI. Representative photos at low magnifications (**upper and middle panel**) are presented. The boxed area is magnified (**lower panel**). Bars: 200 μm (**upper and middle**); 50 μm (**lower**).

Figure 2. Girdin is upregulated in vein grafts with poor run-off. **A**, Representative elastica van Gieson-stained sections of vein grafts harvested at indicated days post-operation. Lower

arrowheads indicate the internal elastic lamina. Bar: 100 μm. **B**, Western blot analyses showed Girdin expression in rabbit whole-vein grafts with poor run-off or normal run-off. Bar graph shows Girdin protein levels (relative to β-actin) determined by densitometry in whole vein grafts harvested at the indicated days post-operation. Girdin level in intact veins was defined as 1 (error bars: SEM; n = 3 for each group; *P < .05).

Figure 3. Girdin is required to rearrange actin cytoskeletons in venous SMCs. **A**, Venous SMCs were stained using anti-Girdin antibody and anti-β-actin antibody. Girdin localizes to actin stress fibers. Bar: 20 μm. **B**, Venous SMCs transfected with either control or *Girdin* siRNA (si-rb2) were stimulated with serum to induce stress fiber formation, which was immunostained using anti-β-actin antibody. Bar: 20 μm. **C**, Venous SMCs were stimulated with PDGF-BB (20 ng/ml) for 10 minutes and stained with anti-β-actin antibody. Red dotted lines denote lamellipodia at the leading edge. Bar: 20 μm. **D**, In experiments described in **C**, the increased number of cells with extended lamellipodia formation after PDGF stimulation was quantified (error bars: SEM; n = 3 for each group; *P < .05). Inset shows a schematic illustration of extended lamellipodia formation. Cells with lamellipodia at more than one third (> 120°) of the cell periphery were counted as cells with extended lamellipodia.

Figure 4. Depletion of Girdin inhibits venous SMCs migration and proliferation *in vitro.* **A**, Venous SMCs transfected with either control or *Girdin* siRNA (si-rb2) were cultured in growth medium containing 10% FBS. Cells were allowed to migrate toward the wound for 12

hours. Bar: 300 μm. **B**, Ratio of the migration distance in each group at each time. **C**, Number of cells that had migrated into the wounded area. (**B**, **C**; n = 6 for each group. Three independent experiments were performed. Error bars: SEM; *P < .05. **D**, WST-1 assay was performed to assess cell proliferation. Absorbance of each cell at day 0 was defined as 1. OD, optical density (n = 5; Error bars: SEM; *P < .05).

Figure 5. Depletion of Girdin inhibits intimal hyperplasia in vein grafts *in vivo*. **A**, Western blot analyses show efficacy of the treatment with atelocollagen containing *Girdin* siRNA in whole vein grafts 7 and 14 days post-operation. Bar graph shows relative protein levels of Girdin determined by densitometry. (Error bars: SEM; n = 3; *P < .05). **B**, Representative elastica Van Gieson-stained sections from animals at 4 weeks post-operation with or without *Girdin* siRNA treatment. Representative photos at low (**upper panel**) and high (**lower panel**) magnifications are presented. Bar: 1.0 mm (**upper**), 100 μm (**lower**). **C/D**, Intimal thickness (**C**) and intima/media ratio (**D**) in vein grafts treated with *Girdin* siRNA were significantly reduced at 4 weeks post-operation. (Error bars: SEM; n = 5 for each group; *P < .05).

Figure 6. Cell proliferation and survival in vein grafts post-operation. **A**, Ki-67-immunostained sections of vein grafts treated with either *Girdin* siRNA or control siRNA. Bar: 100 μm. **B**, Percentage of Ki-67+ cells in area inside of vein graft internal elastic lamina 7 days post-operation. **C**, Percentage of Ki-67+ cells in media or intima of vein grafts 28 days post-operation. (Error bars: SEM; Day 7, n = 3; Day 28, n = 5; *P < .05. N.S., not significant).

D, Vein grafts were subjected to TUNEL staining at 7 or 28 days post-operation. Bar: 100 μm. **E**, Percentage of TUNEL+ cells in area inside of vein graft internal elastic lamina 7 days post-operation. **F**, Percentage of TUNEL+ cells in media and intima of vein grafts 28 days post-operation. (Error bars: SEM; Day 7, n = 3; Day 28, n = 5; N.S., not significant).

Supplemental Methods

Western blot analysis

After SDS-electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (Millipore Corporation, Bedford, MA). Membranes were blocked in 5% skim milk in TBS-T buffer (20 mM Tris-HCl [pH 7.6], 137 mM NaCl, 0.1% Tween 20) with gentle agitation and incubated with the primary antibody. After washing the membranes with TBS-T buffer, they were incubated with secondary antibody conjugated to HRP. After washing the membranes, the reaction was visualised by ECL Detection Kit (GE Healthcare, Buckinghamshire, UK) according to manufacturer's instructions.

Immunofluorescence staining

Venous SMCs were plated on culture slides. Cells were fixed with methanol and 4% paraformaldehyde, blocked with 3% BSA in PBS, and stained with anti-β-actin antibody and the rabbit polyclonal anti-Girdin antibody overnight at 4°C. They were then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody and Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (Invitrogen) for 1 h. Fluorescence was examined using confocal laser-scanning microscopy (Fluoview FV500, Olympus, Tokyo, Japan).

In rabbit vein grafts, transverse sections (4 μm) were blocked with Protein Block Serum-Free Ready-to-Use and subsequently incubated with a mouse monoclonal anti-α-SMA antibody and sheep polyclonal anti-Girdin antibody overnight at 4°C. Thereafter, the sections

were incubated with donkey anti-mouse IgG antibody conjugated with Alexa Fluor 488 and anti-sheep IgG antibody conjugated with Alexa Fluor 594 (Invitrogen). DAPI was used as counter stain.

Cell migration assay

Venous SMCs were seeded on 35-mm glass bottom dishes and transfected with either control or *Girdin* siRNA. Confluent cells were scratched with a 200-μL disposable plastic pipette tip and allowed to migrate toward the wound. Wounded cells were incubated with 10% FBS in DMEM at 37°C; digital images were taken with a microscope (IX70, Olympus). Wound closure rates were calculated as percentages of migratory distances of control cells (100%).

WST-1 assay

Venous SMCs (2.0×10^4 cells/well) transfected with either control or *Girdin* siRNA were plated in 96-well plates 48 h after transfection and allowed to proliferate for 2 or 4 days with DMEM containing 10% FBS. WST-1 reagent (10 μL) was added to 100 μL of cell suspension and incubated for 4 h. Absorbance of each well was measured at 450 nm using a microplate reader.

Supplemental figure legends

Supplemental Figure 1. Girdin localizes to venous SMCs in vessel walls of intact veins. Rabbit intact veins were subjected to immunohistochemical analysis using anti-Girdin and anti-α-SMA antibodies (**upper panels**). Note that these veins are dilated with infusion pressure during fixation and vascular walls look thinner than under normal conditions. The boxed areas are magnified (**lower panels**). Bar: 100 μm.

Supplemental Figure 2. Effects of siRNA on Girdin expression in cultured cells. **A**, Venous SMCs were transfected with three types of *Girdin* siRNAs or control siRNA, or not treated (normal). A representative Western blot for Girdin is shown. Relative densitometric intensities of Western blot bands for Girdin are presented as means ± SEM in graph (n = 3). **B**, Stabilized siRNA (siSTABLE) retained inhibitory effects equal to those of unmodified siRNA. (n = 3)

Supplemental Figure 3. Local application of atelocollagen-mediated siRNA. **A**, siRNA mixed with atelocollagen was used to coat external surfaces of vein grafts. CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; VG, vein graft. **B**, There were no significant differences in medial thickness among vein grafts non-treated and treated with *Girdin* siRNA or control siRNA at 4 weeks post-operation. (Error bars: SEM; n = 5 for each group).

Supplemental Figure 4. Immunohistochemical analysis of vein grafts using anti-CD31 antibody. **A**, Representative immunostaining with anti-CD31 antibody. Bar: 100 μm. **B**, Percentage of luminal coverage by endothelial cells (error bars: SEM; n = 3 per each group; N.S., not significant).

Supplemental Figure 5. Girdin expression level in phenotypic modulation in venous SMCs. **A**, Western blot analyses show Girdin protein levels in venous SMCs treated with TGF-β (2.5 μg/L), PDGF-BB (25 μg/L), 10% FBS or both FBS and PDGF-BB for 48 h. Relative densitometric intensities of Western blot bands of Girdin are presented as means ± SEM in the graph. Girdin protein level in serum-starved venous SMCs (serum(–)) was defined as 1 (n = 3). **B**, Morphological changes in venous SMCs with indicated stimulation for 48 h. Bar: 60 μm.