Novel Small Leucine-Rich Repeat Protein Podocan Is a Negative Regulator of Migration and Proliferation of Smooth Muscle Cells, Modulates Neointima Formation, and Is Expressed in Human Atheroma

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**Background**—Smooth muscle cell (SMC) migration and proliferation critically influence the clinical course of vascular disease. We tested the effect of the novel small leucine-rich repeat protein podocan on SMC migration and proliferation using a podocan-deficient mouse in combination with a model of arterial injury and aortic explant SMC culture. In addition, we examined the effect of overexpression of the human form of podocan on human SMCs and tested for podocan expression in human atherosclerosis. In all these conditions, we concomitantly evaluated the Wnt-TCF (T-cell factor) pathway.

**Methods and Results**—Podocan was strongly and selectively expressed in arteries of wild-type mice after injury. Podocan-deficient mice showed increased arterial lesion formation compared with wild-type littermates in response to injury (P<0.05). Also, SMC proliferation was increased in arteries of podocan-deficient mice compared with wild-type (P<0.05). In vitro, migration and proliferation were increased in podocan-deficient SMCs and were normalized by transfection with the wild-type podocan gene (P<0.05). In addition, upregulation of the Wnt-TCF pathway was found in SMCs of podocan-deficient mice both in vitro and in vivo. On the other hand, podocan overexpression in human SMCs significantly reduced SMC migration and proliferation, inhibiting the Wnt-TCF pathway. Podocan and a Wnt-TCF pathway marker were differently expressed in human coronary restenotic versus primary lesions.

**Conclusions**—Podocan appears to be a potent negative regulator of the migration and proliferation of both murine and human SMCs. The lack of podocan results in excessive arterial repair and prolonged SMC proliferation, which likely is mediated by the Wnt-TCF pathway. (Circulation. 2013;128:2351-2363.)

**Key Words:** arteries ■ cell movement ■ cell proliferation ■ extracellular matrix ■ myocytes, smooth muscle

Extracellular matrix (ECM) molecules are highly effective and selective modulators of important cell functions such as migration and proliferation. The small leucine-rich repeat proteins found in the ECM are also potent regulators of cell phenotype. This growing family of small leucine-rich repeat proteins is composed of 5 classes defined by the number of leucine-rich repeats, the N-terminal composition, and the number of exons. Members of class I, biglycan and decorin, are among the best studied ECM molecules in fibrosis and cancer. We cloned podocan, a novel member of the small leucine-rich repeat protein family, which differed in all 3 classifying categories and, as a result, established a new (fifth) class of this protein family. We identified podocan by representational difference analysis of cDNA in HIV-1 transgenic and nontransgenic podocytes. Podocan mRNA and protein expression increases in sclerotic glomerular lesions of HIV-associated nephropathy but is also present, albeit at lower levels, in normal heart and kidney and in smooth muscle cells (SMCs) in vivo and in vitro. Human and murine podocans share a >91% homology. Recently, podocan has also been shown by other investigators to be present in human aortic tissue.

**Clinical Perspective on p 2363**

Given the inhibitory effect of decorin on SMC proliferation and the ability of biglycan to enhance SMC proliferation, we...
hypothesized that podocan could also modulate SMC migration and proliferation. Human atheroma has a varying content of fibrotic tissue depending on the prevailing driving factors of lesion formation, such as hyperlipidemia, smoking, diabetes mellitus, or mechanical injury after percutaneous coronary intervention. The close regulation of SMC migration and proliferation within the intimal space is critical in maintaining a delicate balance between insufficient and excessive plaque repair. When SMC proliferation is too suppressed, the ensuing weakening of the fibrous cap can result in the plaque vulnerability that underlies acute coronary syndrome, and when SMC proliferation is excessive, intimal hyperplasia can follow, such as in restenosis after percutaneous coronary intervention.

Several important SMC growth-regulatory pathways and molecules have been shown to modulate arterial lesion formation, among them platelet-derived growth factor (PDGF) and transforming growth factor-β. Recently, an important developmental pathway, the Wnt-TCF (T-cell factor) pathway, has been implicated in the regulation of SMC proliferation in vitro and in vivo. Wnt activation via its cell surface receptors leads to an increase in nonphosphorylated β-catenin (stable form) and a reduction in phosphorylated β-catenin (form marked for degradation). Their ratio is used as a marker of Wnt activation. Subsequent nuclear translocation of β-catenin, a hallmark of complete Wnt-TCF pathway activation, controls the transcription of multiple target genes that affect cell proliferation, migration, and survival.

To test the effect of podocan on SMC proliferation and arterial response to injury in vivo, we generated mice deficient in podocan and performed a femoral arterial denudation injury as described previously. We also generated primary aortic SMC explant cultures with podocan-deficient and wild-type (WT) genotypes to examine the effects of podocan deficiency on SMC migration and proliferation in vitro. In addition, we overexpressed the human form of podocan deficiency on SMC migration and proliferation and when SMC proliferation is excessive, intimal hyperplasia can follow, such as in restenosis after percutaneous coronary intervention.

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**Methods**

**Generation of Podocan-Deficient Mice**

A podocan-targeting vector was constructed by insertion of a neomycin cassette, which led to the deletion of exons III through VIII, abolishing podocan expression (online-only Data Supplement). After embryonic stem cell (ES cell) transfection, selection of positive ES cells, and blastocyst injection, the resulting chimeric males were crossed with C57/BL6 female mice. Heterozygous offspring were bred to homozygosity. Genotyping was achieved by use of podocan-specific primers in polymerase chain reaction. Mice were housed at the Center for Laboratory Animal Sciences at the Mount Sinai Medical Center, New York, NY. Mice received standard rodent chow (Purina 5015, PMI Nutrition International, Purina Mills, Richmond, VA ) and tap water ad libitum. Procedures and animal care were approved by the Institutional Animal Care and Use Committee and were in accordance with the “Guide for the Care and Use of Laboratory Animals” (National Research Council, Washington, DC, National Academy Press, 1996).

**Endothelial Denudation Injury of Mouse Femoral Artery**

Mice were anesthetized with intraperitoneal pentobarbital sodium (40 mg/kg; Nembutal, Abbott Laboratories). Removal of the endotherium of the common femoral artery with a surgical microscope was achieved by 3 passages of a 0.25-mm angioplasty guidewire (Advanced Cardiovascular Systems, Santa Clara, CA) in 51 podocan-deficient and WT mice. The protocol, as well as the degree of injury applied to the vessel wall, has been standardized, validated, and described in detail in previous studies.

**Tissue Preparation, Histology, and Immunostaining**

Animals were euthanized with intraperitoneal pentobarbital sodium and perfusion fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 100 mm Hg for 10 minutes and their hindlimbs excised en bloc. Animals at the 4- and 6-week time points were injected with bromodeoxyuridine (BrdU; Sigma-Aldrich, St. Louis, MO) 24 hours before they were killed. Specimens were fixed overnight in 4% paraformaldehyde in PBS and decalcified in 10% formic acid. Two 2-mm-thick cross sections were cut from each hindlimb at the level of the femoral injury and processed for paraffin embedding. Sequential sections (4 μm thick) were stained with Masson’s trichrome and hematoxylin-eosin. Immunohistochemistry was performed with polyclonal rabbit antibodies against murine and human podocan (generated in our laboratory; 1:45 and 1:25, respectively), von Willebrand factor (Dako, Glostrup, Denmark; 1:1000), smooth muscle α-actin (Sigma-Aldrich; 1:300), nonphosphorylated β-catenin (Cell Signaling Technology, Danvers, MA; 1:150), anti-BrdU antibody (Accurate Chemical & Scientific Corp, Westbury, NY; 1:400), and Ki-67 (R&D Systems, Minneapolis, MN; 1:150). Slides were quenched with 3% H2O2, blocked with 1% BSA in PBS, and incubated with the primary antibodies at 37°C for 2 hours. After slides were washed in PBS, bound primary antibody was detected with an appropriate biotinylated secondary antibody for 15 minutes at 37°C. Sections were washed in PBS, subjected to reaction with horseradish peroxidase–conjugated streptavidin, developed with 3,3′-diaminobenzidine, and counterstained with hematoxylin. Negative controls were prepared by substitution of primary antibody with the respective control IgG. Double labeling was performed with FITC- and Texas Red–conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) with DAPI counterstaining.

**Computer-Assisted Morphometry**

Investigators blinded to the study design performed the histomorphometric evaluation. A computer-assisted planimetry system was used (Image-Pro Plus, Media Cybernetics, Rockville, MD). Neointima formation was assessed by hematxylin-eosin and Masson’s trichrome staining. SMC density and proliferation (Ki-67 and BrdU labeling) were quantified as 0-actin–positive cells per area and as percentage of Ki-67/BrdU-positive cells from total cells with nuclear counterstaining. No significant interobserver or intraobserver variations were noted.

**Culture of Murine and Human SMCs and Podocan Transfection**

Aortic SMCs were prepared by the explant method from podocan-deficient mice or WT littermates. Briefly, the aortas were freed of any connective tissue and adherent perivascular fat, the endothelial cell layer was removed, and the arteries were cut into 3-mm rectangular pieces. The pieces were placed in DMEM (Gibco, Life Technologies, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS), 100 U/mL penicillin, 100 g/mL streptomycin, and 0.25 μg/mL amphotericin B in a humidified atmosphere of 5% CO2 and 95%
air at 37°C. The SMCs generated exhibited a typical hill-and-valley growth pattern, and morphological examination and smooth muscle α-actin staining confirmed the cell type. Human primary aortic SMCs were obtained commercially (PromoCell, Heidelberg, Germany) and seeded in 10-mL culture flasks. Medium was replaced every other day. All SMCs were serially passaged before they reached conflu-ence, and all experiments were performed on SMCs from passages 2 to 4. Cells were washed 3 times with HBSS and rendered quiescent in serum-free DMEM for 24 hours before experiments. Podocan transfection experiments were performed according to standard protocols. In brief, the expression vectors encoding the full-length mouse/human podocan protein (pCDNA3.1-m/h podocan) and control vector (pCDNA3.1) were transfected into SMCs with FuGENE 6.0 (Roche Diagnostics GmbH, Mannheim, Germany). The cells were harvested at 48 hours after transfection for evaluation of SMC proliferation and migration. Protein analyses confirmed the expression of podocan in podocan-deficient cells transfected by pCDNA3.1-mPodocan and in human SMCs transfected by pCDNA3.1-hPodocan.

**Cell Proliferation Assay**

To assess the proliferation of SMCs, cells were trypsinized, washed 2 times with PBS, and added to gelatin-coated 96-well plates at a density of 5×10^3 cells/well in DMEM containing either 10% FBS or recombinant PDGF (R&D Systems). After culture for 72 hours, cell number was assessed with the MTS assay (Promega, Madison, WI). For human SMCs treated with either podocan-expressing vector or empty vector and for untreated cells, a colorimetric BrdU-incorporation assay (Roche) was used.

**Cell Migration Assay**

The migration of SMCs was examined with a colorimetric cell migration assay (Chemicon, Temecula, CA) based on the Boyden chamber principle using inserts with a pore size of 8 μm. SMCs were trypsinized, washed 2 times with PBS, resuspended in 1% FBS in DMEM, and added to the top wells (2.5x10^4 cells/300 μL). DMEM with 10% FBS or recombinant mouse PDGF (R&D Systems) was added to the bottom chamber. After 6 hours at 37°C, nonmigrating cells were scraped from the upper surface of the filter. Cells on the bottom surface were incubated with Cell Stain Solution (Chemicon, Temecula, CA), then subsequently extracted and detected by spectrophotometry (absorbance at 560 nm).

**Wnt-TCF Pathway Evaluation**

For evaluation of Wnt-TCF pathway–related protein expression, SMC lysates were prepared for protein electrophoresis and Western blotting with RIPA (radioimmunoprecipitation assay) lysis buffer (Santa Cruz Biotechnology, Dallas, TX) and PARIS kit (Ambion, Life Technologies). Imaging and analysis were performed with a FluorChem 8800 system and AlphaEasy FC software (Alpha Innotech/Protein Simple, Santa Clara, CA). Specific antibodies against phosphorylated and nonphosphorylated β-catenin (Cell Signaling Technology) were used for Western blotting. To determine Wnt-TCF pathway activation by measuring β-catenin/Tcf/Lef-1 transcriptional activity directly, we used a luciferase-based transcriptional reporter assay. TOPflash/FOPflash plasmids (Upstate Biotechnology, Lake Placid, NY) were transfected into cultured SMCs. Cells were cotransfected with pRL-SV40 (Promega) as internal control. Forty-eight hours later, reporter luciferase activity was measured by dual luciferase reporter assay (Promega) and normalized to Renilla luciferase activity. TCF reporter luciferase activity was represented by the ratio of TOPflash and FOPflash luciferase activity. All in vitro experiments were performed in triplicate and repeated a minimum of 3 times. See the online-only Data Supplement for a more detailed description of this assay.

### Table. Coronary Atherectomy Samples: Patient and Lesion Characteristics

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Ath indicates atherectomy; LAD, left anterior descending coronary artery; Pre/Post, before and after angioplasty or atherectomy; PTCA, percutaneous transluminal coronary angioplasty; RCA, right coronary artery; and RCx, right circumflex coronary artery.
Origin and Analysis of Human Arterial Specimens
Paraffin blocks of formalin-fixed atherosclerotic carotid plaque tissue were obtained from carotid endarterectomy specimens (n=7). Use of excess anonymous surgical pathology tissue was approved by the institutional review board. Percutaneous directional atherectomy was performed in patients presenting with stable angina attributed to the presence of stenotic primary atherosclerotic lesions or restenotic lesions after previous balloon angioplasty or atherectomy (2.2–20 months after the initial interventional procedure). Tissue samples were obtained by atherectomy from a total of 18 coronary target lesions, including 7 restenotic and 11 primary lesions (angiographic stenosis degree >75%) as shown in the Table. The origin of these atherectomy samples was the left anterior descending artery in 12 cases, the right coronary artery in 5 cases, and the circumflex coronary artery in 1 case. Restenosis was defined according to previously reported clinical and angiographic criteria.28,29 Informed consent for the analysis of tissue samples was obtained from all patients before revascularization. Immediately after percutaneous atherectomy, all specimens were fixed in 4% paraformaldehyde in PBS. Subsequently, specimens were processed for paraffin embedding. Sequential sections (4 µm thick) were cut and stained with Masson’s trichrome and hematoxylin-eosin. Immunohistochemistry was performed with polyclonal rabbit antibodies against human podocan (generated in our laboratory; 1:45), smooth muscle α-actin (Sigma; 1:300), and nonphosphorylated β-catenin (Cell Signaling Technology; 1:150). Hematoxylin- and Masson’s trichrome–stained sections allowed for the counting of cells in the intima; adjacent medial areas of the vessels were not analyzed. Assessment of cell density and expression of podocan and nonphosphorylated β-catenin was performed with a computer-assisted morphometry system as described above. Nuclei were counted per area and used to calculate the cell density per cubic millimeter; podocan expression was measured as percentage of intimal area covered by podocan staining; and expression of nonphosphorylated β-catenin was measured as a percentage of intimal cells with nuclear nonphosphorylated β-catenin labeling. Ten randomly selected intimal areas, each encompassing 0.04 mm², were assessed per tissue sample as described previously.30

Statistical Analysis
SPSS/PC+ software (IBM, Armonk, NY) was used for data analysis. Data are shown as mean±SEM (in vivo data) and as mean±SD (in vitro data). Two-way ANOVA testing was used to evaluate neointima area, reendothelialization, SMC density, and expression of Ki-67/BrdU with podocan-deficient and WT genotype. After testing for normal distribution and equality of variances with the Levene test, the F test, the t test, and the t test. Probability values were 2-tailed and corrected for ties. P <0.05 was considered significant.

Results
Expression of Podocan in Injured Mouse Femoral Artery
In noninjured femoral arteries of WT animals, podocan expression could not be detected by immunostaining (Figure 1A and 1D). In contrast, podocan was found consistently in arteries of WT mice after injury. Podocan deposition was seen surrounding medial and neointimal SMCs (Figure 1B and 1E). Injured arteries of podocan-deficient mice were completely devoid of podocan, as expected, which confirms the specificity of the podocan antibody (Figure 1C and 1F). The complete time course analysis of podocan expression in WT arteries using antibodies for podocan and α-actin showed barely detectable podocan staining at 1 week in α-actin–positive media (Figure 1G and 1H). At 2 weeks after injury, a strong, albeit patchy, medial podocan expression appeared (arrows). Of note, podocan was also seen in early neointima and showed, as expected, no podocan labeling, serving as negative control for podocan immunostaining. Podocan expression at 4 weeks after injury was almost undetectable in the media, whereas α-actin was expressed strongly (Figure 1I and 1J). At 2 weeks, much stronger, albeit patchy, medial podocan expression appeared (arrows). Of note, podocan was also seen in early neointima. Magnification, ×200; scale bar, 50 µm. K and L. At 4 weeks, podocan expression (arrows) remained strong in neointima and media in close spatial association with α-actin signals. Magnification, ×200; scale bar, 50 µm. K and L.
Effect of Podocan on Arterial Response to Injury

We examined the effect of podocan genotype on arterial response to injury in WT (n=27) and podocan-deficient (n=28) mice. At 1 and 2 weeks, no significant difference in neointima size was found between the groups (1 week: 2.0±0.9 versus 1.8±0.8×10^3 mm^2, P=NS; 2 weeks: 3.8±1.0 versus 2.9±0.9×10^3 mm^2, P<0.05; Figure 2A–2F and 2M). At 4 weeks, however, the neointima area was significantly greater with podocan-deficient genotype than with WT (11.6±1.8 versus 4.4±1.3×10^3 mm^2, P<0.05; Figure 2C, 2F, and 2M). The neointima to media ratio was also increased with podocan-deficient genotype at 4 weeks (3.0±0.4 versus 1.1±0.15; P<0.01). SMC density did not show a significant difference between groups early after injury (1 week: 2078±978 versus 1958±347×10^3 cells/mm^2, P=NS; 2 weeks: 8822±2078 versus 7823±978×10^3 cells/mm^2, P=NS; Figure 2G–2L and 2N). At 4 weeks, however, SMC density of neointima was significantly increased with podocan-deficient genotype (18±3% versus 12±2%, P=NS; Figure 2A–2F and 2M). Measuring proliferation by BrdU incorporation confirmed the increased SMC proliferation found with podocan-deficient genotype (18±3% versus 2±2%, P<0.05; Figure 4G–4K).

Effect of Podocan on Migration and Proliferation in Mouse and Human SMCs

In WT aortic explants, there was no cellular outgrowth at 3 days in any of the 8 samples (Figure 5A). In contrast, at the edge of podocan-deficient aortic explants, SMC outgrowth was visible in 6 of 8 samples at 3 days, which indicates early SMC outgrowth (Figure 5B). Subsequently, we compared migration of the cultured SMCs and found that...
podocan-deficient SMCs grown in 10% FBS migrated significantly faster than WT cells (0.73±0.06 versus 0.55±0.03, P<0.05; Figure 5C). Podocan-deficient SMCs also grew at a significantly greater rate than WT cells when cultured in 10% FBS or in response to recombinant PDGF (10 ng/mL; 10% FBS: 0.76±0.03 versus 0.69±0.03, P<0.05; 10 ng/mL PDGF: 1.01±0.03 versus 0.89±0.03, P<0.05) as measured by the MTS assay (Figure 5D). In an attempt to restore the WT SMC phenotype, we transfected podocan-deficient SMCs with podocan-expressing vector. Podocan synthesis was induced in podocan-deficient SMCs treated with podocan-expressing vector as confirmed by Western blot (data not shown). Proliferation in both 10% FBS (0.35±0.01 versus 0.40±0.01; P<0.05) and with PDGF stimulation (0.49±0.02 versus 0.65±0.02; P<0.05) was significantly reduced compared with empty vector treatment and approached that seen with WT cells (Figure 5E). Moreover, in human SMCs treated with human podocan-expressing vector, podocan was highly enriched compared with vector control and untreated SMCs as assessed by Western blot (Figure 5I). Podocan overexpression resulted in a 29% reduction of SMC migration (0.40±0.08 versus 0.56±0.09, P<0.05; Figure 5J). Using a BrdU-incorporating assay, we also found a time-dependent inhibition of SMC proliferation up to 32% (at 24 hours) with podocan overexpression (0.15±0.01 versus 0.22±0.01, P<0.05 at 24 hours; Figure 5K). All quantitative data in this section represent units of optical density resulting from spectrophotometric measurements.

Effect of Podocan on the Wnt-TCF Pathway in Mouse and Human SMCs

We found a reduction in phosphorylated and an increase in nonphosphorylated β-catenin in podocan-deficient SMCs compared with WT, indicative of Wnt pathway activation (Figure 5F). To confirm the increase in transcriptional Wnt activity in podocan-deficient SMCs, we performed TOPflash/FOPflash reporter assays. The TOPflash/FOPflash assays showed >2-fold enhancement in nuclear β-catenin/Tcf/Lef-1 transcriptional activity in podocan-deficient SMCs, which confirmed activation of Wnt signaling (Figure 5G). When we treated podocan-deficient SMCs with β-catenin small inhibitory RNA (siRNA), we observed a significant suppression of nonphosphorylated β-catenin compared with control siRNA treatment (data not shown). Of note, β-catenin RNA
silencing resulted in inhibition of SMC proliferation comparable to the inhibition achieved by WT podocan gene transfection into podocan-deficient SMCs (Figure 5H). Of note, the in vivo expression of nonphosphorylated (stable) β-catenin was also strongly increased in podocan-deficient neointima compared with WT (2 weeks: 10±3% versus 4±2%, P>0.05; 4 weeks: 38±8% versus 8±3%, P<0.05; Figure 6A–6F and 6L). Importantly, podocan-deficient SMC in the neointima displayed nuclear nonphosphorylated β-catenin staining indicative of nuclear β-catenin translocation, a hallmark of Wnt activation (Figure 6G–6I). Conversely, enrichment of the human form of podocan in human SMCs by treatment with podocan-expressing vector resulted in a significant increase in phosphorylated β-catenin over nonphosphorylated β-catenin seen by Western blot (Figure 5L), consistent with Wnt-TCF pathway suppression.

Podocan and Wnt-TCF Pathway in Human Atheroma

In atherectomy samples from patients with primary stable atherosclerosis (n=11), podocan expression was abundant (Figure 7). In restenotic lesions, podocan expression was decreased significantly, with 8±2% of intimal area compared with 30±4% in primary coronary lesions (P<0.05). In contrast, intimal cell density was increased significantly in restenotic compared with primary coronary lesions, with 632±107 versus 195±40 cells per mm² (P<0.05). Of note, the expression of nonphosphorylated β-catenin was strongly increased in restenotic lesions compared with primary lesions (22±5% versus 5±1%, P<0.05). Importantly, nuclear staining of nonphosphorylated β-catenin, indicative of nuclear translocation, was observed in restenotic lesions (Figure 8). Immunofluorescence labeling showed a colocalization of nonphosphorylated β-catenin and smooth muscle α-actin in hyperplastic areas of restenotic lesions (Figure 8). In all coronary lesions, we observed an inverse correlation between the extent of podocan deposition and nonphosphorylated β-catenin expression (r=-0.78, P<0.05) and a strong positive correlation between the expression of nonphosphorylated β-catenin and intimal cell density (r=0.94, P<0.05). Of note, in both lesion types, staining with an isotype control antibody that matched the podocan antibody did not show any staining, excluding nonspecific labeling or autofluorescence artifact (Figure 7).

Discussion

The results of the present study suggest that the novel small leucine-rich repeat protein podocan is a key regulator of the SMC response after arterial injury. Lack of podocan expression with podocan-deficient genotype resulted in late and
inhibitory RNA.

PDGF, platelet-derived growth factor; and siRNA, small inhibitory RNA.

OD 490, optical density 490 nm; OD 588, optical density 588 nm; bromodeoxyuridine; eGFP, enhanced green fluorescent protein; L

K

J

I

H

G

F

E

D

C

B

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K

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Figure 5. Effect of wild-type and podocan-deficient genotype on murine aortic smooth muscle cells (SMC). A and B, Aortic explant culture. A, The edge of wild-type aortic explant showed no SMC outgrowth at day 3. Magnification, ×400; scale bar, 50 μm. B, In contrast, numerous SMCs were seen at the edge of a podocan-deficient aortic explant at the same time point. Magnification, ×400; scale bar, 50 μm. C–E, SMC migration and proliferation. C, SMC migration was increased with podocan-deficient genotype compared with wild-type in a colorimetric test based on the Boyden chamber principle (independent-sample t test). D, Podocan-deficient SMCs also grew faster as measured by the MTS assay (independent-sample t test). E, Podocan-deficient SMCs transfected with podocan vector slowed their growth to wild-type-level (independent-sample t test). F, Wnt-TCF pathway activation. F, In SMCs with podocan-deficient genotype, the ratio of phosphorylated to nonphosphorylated β-catenin was reversed as seen by Western blot. G, Transcriptional activity of Wnt-TCF pathway measured directly by TOPflash/FOPflash assay was also increased with podocan-deficient genotype. H, Podocan-deficient SMCs treated with small inhibitory RNAs to β-catenin showed inhibition of growth. I–L, Effect of podocan overexpression on human aortic SMCs. I, Western blot confirmed overexpression of the human form of podocan. Podocan in control and empty vector–treated SMCs was below the detection threshold. J, SMC migration was reduced by 29% with podocan overexpression. K, SMC proliferation was reduced by 32% with podocan overexpression. L, SMC–overexpressing podocan showed an increase in phosphorylated β-catenin on Western blot compared with nontreated or empty vector–treated SMCs, which indicates Wnt-TCF pathway suppression. BRDU indicates bromodeoxyuridine; eGFP, enhanced green fluorescent protein; OD 490, optical density 490 nm; OD 588, optical density 588 nm; PDGF, platelet-derived growth factor; and siRNA, small inhibitory RNA.

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The possible role of podocan as a physiological inhibitor of the SMC migratory and proliferative response is suggested by several observations. Podocan is not expressed constitutively at high levels by vascular SMCs at baseline. In the absence of injury, podocan expression could not be detected by immunohistochemistry. Only after arterial injury was podocan gradually expressed at increasing and robust levels in the media and neointima (between 2 and 4 weeks after injury). Of note, in podocan-deficient mice, arterial lesion formation was affected specifically between 2 and 4 weeks after injury compared with WT mice. This is precisely the time when ECM synthesis and podocan deposition typically take place in the neointima. Occurring after the initial stages of cell adhesion and cell recruitment, this phase of ECM build up and remodeling has been described by several investigators studying different models of arterial injury. Consistent with the postulated inhibitory regulatory function of podocan, we found an increase in proliferation in neointima devoid of podocan in animals with podocan-deficient genotype as late as 4 weeks after injury. This finding is unusual, because the natural history of arterial wall cell proliferation in most models peaks during the first 2 weeks and tapers off at later time points.
In addition to the late increase in proliferation, we also found a late increase in SMC density at 4 weeks in the podocan-deficient neointima. Previous studies reported that by 4 weeks, SMC density usually declines because of a decreased rate of SMC proliferation and ongoing ECM synthesis. Even as late as 6 weeks after arterial injury, robust SMC proliferation was still evident in the neointima of podocan-deficient mice.

To extend these in vivo observations and to better define the podocan genotype effect, we explanted SMCs from podocan-deficient and WT aortas. The podocan genotype determined the rate of migration and proliferation of the cultured SMCs with strongly enhanced migration and proliferation of SMCs that lacked podocan expression. Transfection of podocan-deficient SMCs with WT podocan decreased proliferation to WT levels, essentially “normalizing” the podocan-deficient phenotype and indicating a specific podocan effect. To test whether podocan has a true inhibitory effect on podocan-competent WT cells as well and to assess the function of the human isoform of podocan, we overexpressed human podocan in human SMCs. Consistent with our observations in murine SMCs, transfection of SMCs with the human form of podocan significantly reduced migration and proliferation.

To define the possible mechanism by which podocan exerts its effect on SMCs, we probed for changes in the Wnt-TCF pathway, which has a central role in controlling proliferation and migration of cells. Of note, we detected significant and corresponding alterations in the Wnt-TCF pathway in each of these 2 different experimental settings. Cultured murine SMCs with podocan-deficient genotype showed increased Wnt-TCF pathway activation compared with WT SMCs. Conversely, human podocan-competent SMCs that overexpressed podocan showed significantly decreased Wnt-TCF pathway activation. These corresponding changes were verified by 2 independent and established methods of measuring Wnt-TCF pathway activation (the relevant methodology is further outlined in the online-only Data Supplement). Further evidence for an important role of the Wnt-TCF pathway in mediating podocan-related effects on SMCs also stems from the observation that β-catenin siRNA treatment was capable of normalizing increased...
Figure 7. Expression of podocan in human atheroma: Primary carotid atheroma (A–D), primary coronary lesion (E, F, I, J, M, N, Q, R, U, and V), and restenotic coronary lesion (G, H, K, L, O, P, S, T, W, and X). A–D. Podocan immunostaining. An antibody specific for the human form of podocan yielded strong brown labeling in the intima of carotid atheroma (A and C). Magnification, ×100; scale bar, 50 μm. Matching isotype staining on adjacent section showed no labeling (B and D).

E–G. Combined Masson-elastin (CME) staining. Comparison of the histoarchitecture of primary and restenotic coronary lesions showed distinct differences. E, Spindle-shaped smooth muscle cells were surrounded by large spaces of extracellular matrix at a rather low cell density in primary lesions. G, In restenotic tissue, abundant numbers of smooth muscle cells were tightly clustered and surrounded by a comparatively smaller extracellular matrix space. Magnification, ×50, ×100, and ×200; scale bar, 50 μm.

F–V. Two versions of podocan and smooth muscle cell double labeling. F and H, Smooth muscle α-actin (FITC) and podocan (Texas Red) double labeling. Low-power magnification images revealed the inverse relation between the degree of intimal podocan labeling (red) and the density of intimal smooth muscle cells (green) in primary (F) compared with restenotic (H) coronary plaque tissue. J, M, N, R, U, and V, Smooth muscle α-actin (Texas Red) and podocan (FITC) double labeling. With reversed double labeling, higher-power magnification images confirmed that large extracellular matrix spaces in primary lesions surrounding the red-labeled smooth muscle cells were enriched with podocan, as shown by extensive green labeling (arrows). L, O, P, T, W, and X. In contrast, green podocan labeling (arrows) in restenotic tissue covered a much smaller area and was restricted to the immediate vicinity of red-labeled smooth muscle cells. Magnification, ×200 and ×1000; scale bar, 50 μm.

I, Q, K, and S. Corresponding intimal locations in adjacent serial sections were also shown by light microscopy and CME staining. Magnification, ×1000; scale bar, 50 μm. Of note, in both lesion types, staining with an isotype control antibody that matched the podocan antibody did not yield any green signals (z, I–IV).

Y. Comparison of podocan expression (% area) and smooth muscle cell density (cells per mm²) in primary and restenotic coronary lesions; (independent-sample t test).
podocean-deficient SMC proliferation in a similar fashion as the restoration of podocan expression with podocan transfection did.

Returning to the in vivo physiology of the arterial response to injury, we wanted to test whether matching alterations in the Wnt-TCF pathway could also be found in the neointima with podocan-deficient and WT genotypes. Interestingly, we observed a strong increase in the expression of the nonphosphorylated form of β-catenin in the neointima with podocan-deficient genotype. Podocan-deficient neointimal SMCs exhibited a nuclear staining pattern suggestive of nuclear translocation of β-catenin and hence indicative of Wnt-TCF pathway activation. The peak of this Wnt-TCF pathway activation in neointimal SMCs coincided precisely with the late rise in SMC proliferation in podocan-deficient neointima. As a final step, we set out to determine the relevance of podocan and its effects on the Wnt-TCF pathway for the pathophysiology of intimal hyperplasia in humans.

To this end, we examined podocan expression in human coronary restenosis and compared it with podocan expression in primary coronary lesions along with the same marker of Wnt-TCF pathway–related molecules in human vascular lesions provides further evidence for a possible role of podocan as a physiological inhibitor of the SMC migratory and proliferative response in the arterial response to percutaneous coronary intervention in patients. Its effects on SMCs and its ability to modulate, at least in part, the Wnt-TCF pathway renders podocan a novel therapeutic target for better controlling arterial repair. Further experimental work is needed to delineate the molecular interactions of podocan with Wnt-TCF pathway–related molecules and its effects on other cell types.

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Disclosures
None.

References


**CLINICAL PERSPECTIVE**

Smooth muscle cells (SMCs) critically influence the clinical course of vascular disease. The close regulation of SMC migration and proliferation within the intimal space is critical in maintaining a delicate balance between insufficient and excessive atherosclerotic plaque repair. When SMC proliferation is too suppressed, the ensuing weakening of the fibrous cap can result in the plaque vulnerability that underlies acute coronary syndrome, and when SMC proliferation is excessive, intimal hyperplasia can follow, such as in restenosis after percutaneous coronary intervention. Although stents have vastly improved the recoil and constrictive remodeling component of restenosis, the problem of accelerated intimal SMC growth has remained. The current approach of delivering stents that release nonspecific agents that promote cell death or inhibition of proliferation has been successful in reducing the need for recurrent vascular interventions; however, this success comes at the expense of delaying vascular healing, the ultimate long-term clinical impact of which is still being evaluated. Short- and long-term negative effects on the healing arterial wall, such as delayed reendothelialization, increased inflammation, and enhanced thrombogenicity, are undisputed. These side effects of drug-eluting stents are being masked by prolonged and aggressive antiplatelet therapy, which exposes patients, especially the elderly, to increased bleeding risks, complicates clinical decision making through fear of too early treatment cessation, demands rigorous patient compliance, and is costly. These issues are not trivial in daily clinical practice. The possible role of podocan as a novel selective inhibitor of the SMC response and the Wnt-TCF pathway opens the door to modulation of vascular SMCs in a smarter and more physiological way.
Novel Small Leucine-Rich Repeat Protein Podocan Is a Negative Regulator of Migration and Proliferation of Smooth Muscle Cells, Modulates Neointima Formation, and Is Expressed in Human Atheroma

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SUPPLEMENT MATERIAL
Wnt-TCF pathway assay description:

The wnt/beta-catenin/TCF/Lef-1 signal transduction pathway has been implicated in cell fate decisions, migration, proliferation and survival of cells including the pathophysiology of many types of cancer [1]. Binding of secreted wnt-protein to its membrane-bound receptor complex, composed of a member of the frizzled receptor family and the co-receptor LDL-receptor related protein (LRP)-5 or LRP-6 results in the inhibition of phosphorylation of beta-catenin by glycogen synthase kinase. Unphosphorylated beta-catenin remains stable, accumulates in the cytoplasm, and translocates into the nucleus, where it can activate target gene expression through interaction with the transcription factors T-cell factor (TCF) and lymphoid enhancer factor-1 (Lef-1) [1].

Cellular Wnt-TCF pathway activity can be assessed by using the so called TOP/FOP FLASH reporter assay, which measures TCF transcriptional activity. In the TOP-FLASH plasmid, luciferase is driven by functional TCF binding sites, whereas the FOP-FLASH plasmid has mutated, non-functional TCF binding sites and serves as a negative control; the TCF transcriptional activity can be taken as a ratio of TOP-FLASH to FOP-FLASH luciferase-mediated signals. The first time use of TOP-FLASH and FOP-FLASH reporter plasmids has been published initially in 2000 [2] and the assay has ever since been used widely to evaluate Wnt-TCF pathway activity in many different cell types and experimental conditions.

References:

Generation and evaluation of podocan -/- mice:

**Targeting strategy (a):** Targeting vector for the podocan locus with the neomycin resistance cassette (top), restriction map of the genomic region of the wild type podocan allele (middle) and the mutant locus resulting from homologous recombination (bottom).

**Southern blot of offspring from a heterozygote cross (b):** A restriction digest with BclI yielded a 7.18-Kb wild type and 5.10-Kb recombinant band.
**Genotyping (c):** Extraction of mouse tail genomic DNA and use of the podocan and neomycin primers for PCR. PCR with the two primers revealed 0.5 Kb and 1.5 Kb fragments from WT and mutant alleles.

**Expression Analysis (d and e):** (d) RT-PCR of RNA extracted from podocan deficient (-/-), heterozygous (+/) and wild type (+/+) mouse kidneys. (e) Western blots comparing podocan expression in various tissues of podocan deficient and wild type mice with beta-actin loading control.