The 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

Running title: Hutter et al.; Podocan and Smooth Muscle Cells

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Abstract

Background—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 SMC and tested for podocan expression in human atherosclerosis. In all these conditions we evaluated concomitantly the Wnt-TCF-pathway.

Methods and Results—Podocan was strongly and selectively expressed in arteries of WT mice after injury. Podocan-/- mice showed increased arterial lesion formation as compared to WT littermates in response to injury (P<0.05). Also, SMC proliferation was increased in arteries of podocan-/- mice compared to WT (P<0.05). In vitro, migration and proliferation were increased in podocan-/- SMC and were normalized by transfection with the WT podocan gene (P<0.05). In addition, upregulation of the Wnt-TCF-pathway was found in SMC of podocan-/- mice both in vitro and in vivo. On the other hand, podocan overexpression in human SMC 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 SMC. The lack of podocan results in excessive arterial repair and prolonged SMC proliferation, which likely is mediated by the Wnt-TCF-pathway.

Key words: extracellular matrix, smooth muscle cell, arterial injury, migration, proliferation, Arterial repair, Podocan, vascular smooth muscle
Introduction

Extracellular matrix (ECM) molecules are highly effective and selective modulators of important cell functions such as migration and proliferation \(^1\)-\(^4\). The small leucine-rich repeat proteins (SLRP) found in the ECM are also potent regulators of cell phenotype \(^5\). This growing family of SLRP’s is comprised 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 \(^5\)-\(^10\). We cloned podocan, a novel member of the SLRP family, which differed in all three 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 non-transgenic podocytes \(^11\). Podocan mRNA and protein expression increases in sclerotic glomerular lesions of HIV-associated nephropathy (HIVAN) but is also present albeit at lower levels in normal heart, kidney and in smooth muscle cells (SMC) in vivo and in vitro \(^12\). Human and murine podocan share a greater than 91% homology \(^11\). Recently, podocan has also been shown by other investigators to be present in human aortic tissue \(^13\).

Given the inhibitory effect of decorin on SMC proliferation and the capability of biglycan to enhance SMC proliferation, we hypothesized that podocan could also modulate SMC migration and proliferation \(^9\),\(^14\)-\(^17\). Human atheroma has a varying content of fibrotic tissue depending on the prevailing driving factors of lesion formation such as hyperlipidemia, smoking, diabetes or mechanical injury post PCI \(^18\),\(^19\). 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 plaque vulnerability underlying acute coronary
syndrome and when SMC proliferation is excessive, intimal hyperplasia can follow such as in restenosis post PCI \textsuperscript{20,21}.

Several important SMC growth-regulatory pathways and molecules have been shown to modulate arterial lesion formation – among them PDGF and TGF-beta \textsuperscript{3,22}. Recently, an important developmental pathway - the Wnt-TCF-pathway - has been implicated in the regulation of SMC proliferation \textit{in vitro} \textsuperscript{23,24} and also \textit{in vivo} \textsuperscript{25}. Wnt activation via its cell surface receptors leads to an increase in non-phosphorylated beta-catenin (stable form) and a reduction in phosphorylated beta-catenin (form marked for degradation). Their ratio is used as a marker of Wnt-activation. Subsequent nuclear translocation of beta-catenin – a hallmark of complete Wnt-TCF-pathway activation - controls the transcription of multiple target genes affecting cell proliferation, migration and survival \textsuperscript{23,24}.

To test the effect of podocan on SMC proliferation and arterial response to injury \textit{in vivo}, we generated mice deficient in podocan and performed a femoral arterial denudating injury as previously described \textsuperscript{26,27}. We also generated primary aortic SMC explant cultures with podocan-/- and WT genotypes to examine the effects of podocan deficiency on SMC migration and proliferation \textit{in vitro}. In addition, we overexpressed the human form of podocan to assess the effect of increased amounts of podocan on human SMC. To further determine the relevance of podocan for human arterial lesion formation we also tested for podocan expression in different forms of human atherosclerosis. In all these conditions we concomitantly examined the Wnt-TCF-pathway.

\textbf{Methods}

\textbf{Generation of Podocan Deficient Mice}

A podocan-targeting vector was constructed by inserting a neomycin cassette, which led to the
deletion of exons III through VIII, abolishing podocan expression (see also Supplement section).

After 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 using podocan-specific primers in PCR. Mice were housed at the Center for Laboratory Animal Sciences at The Mount Sinai Medical Center, New York. Mice received standard rodent chow (Nutrition International) 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, D.C.: National Academy Press 1996).

**Endothelial Denudation Injury of Mouse Femoral Artery**

Mice were anesthetized with intra-peritoneal pentobarbital sodium (40 mg/kg) (Nembutal®, Abbott Laboratories). Removal of the endothelium of the common femoral artery using a surgical microscope was achieved by 3 passages of a 0.25 mm angioplasty guide wire (Advanced Cardiovascular Systems) in 51 podocan-/− 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 26, 27.

**Tissue Preparation, Histology and Immunostaining**

Animals were sacrificed 1, 2, 4, and 6 weeks after arterial injury and perfusion-fixed with 4% paraformaldehyde (PFA) 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 BRDU (Sigma-Aldrich) 24 hours prior to sacrifice. Specimens were fixed overnight in 4% PFA 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 (4μm 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 lab, 1:45 and 1:25, respectively), von Willebrand Factor (Dako; 1:1000), smooth muscle alpha-actin (Sigma; 1:300), non-phospho beta-catenin (Cell Signaling; 1:150), anti-BRDU antibody (Accurate; 1:400) and Ki-67 (R&D Systems; 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 washing in PBS, bound primary antibody was detected using an appropriate biotinylated secondary antibody for 15 minutes at 37°C. Sections were washed in PBS, reacted 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 using FITC- and Texas Red-conjugated secondary antibodies (Jackson Immuno Laboratories) 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). Neointima formation was assessed by H&E and Masson’s trichrome staining. SMC density and proliferation (Ki-67 and BRDU labeling) were quantified as alpha-actin-positive cells per area and as percentage Ki-67/BRDU-positive cells from total cells with nuclear counterstaining. No significant inter- or intra-observer variations were noted.

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

Aortic SMCs were prepared by the explant method from podocan-/- mice or WT littermates. Briefly, the aortas were freed of any connective tissue and adherent peri-vascular fat, the
endothelial cell layer was removed, and the arteries were cut into approximately 3mm rectangular pieces. The pieces were placed in DMEM (Gibco) supplemented with 20%FBS, 100 U/ml penicillin, 100g/ml streptomycin and 0.25μg/ml amphotericin B in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The SMC generated exhibited a typical “hill and valley” growth pattern and morphological examination and smooth muscle alpha-actin staining confirmed the cell type. Human primary aortic SMC were obtained commercially (Promo Cell) and seeded in 10 ml culture flasks. Medium was replaced every other day. All SMCs were serially passaged before reaching confluence, and all experiments were performed on SMCs from passages 2 to 4. Cells were washed three times with HBSS and rendered quiescent in serum free DMEM for 24 hours prior to 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/hPodocan) and control vector (pCDNA3.1) were transfected into SMC using Fugene 6.0 (Roche). The cells were harvested at 48 h post transfection for evaluation of SMC proliferation and migration. Protein analyses confirmed the expression of podocan in podocan +/- cells transfected by pCDNA3.1-mPodocan and in human SMC 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 5x10³ cells/well in DMEM containing either 10% FBS or recombinant PDGF (R&D Systems). After culture for 72 hours, cell number was assessed using the MTS assay (Promega). For human SMC 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 using a colorimetric cell migration assay (Chemicon) 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, non-migrating cells were scraped from the upper surface of the filter. Cells on the bottom surface were incubated with Cell Stain Solution, 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 using RIPA lysis buffer (Santa Cruz Biotech.) and PARISTM Kit (Ambion). Imaging and analysis were performed by FluorchemTM 8800 system and AlphaEasy FC software (Alpha Innotech). Specific antibodies against phosphorylated and non-phosphorylated beta-catenin (Cell Signaling) were used for Western-blotting. To determine Wnt-TCF pathway activation by measuring beta-catenin/Tcf/Lef-1 transcriptional activity directly, we used a Luciferase-based transcriptional reporter assay. TOPflash/FOPflash plasmids (Promega) were transfected into cultured SMCs. Cells were co-transfected with pRL-SV40 (Promega) as internal control. 48 h 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 triplicates and repeated a minimum of three times. See also the Supplement section for a more detailed description of this assay.
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 to 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 Table 1. 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. Informed consent for the analysis of tissue samples was obtained from all patients prior to revascularization. Immediately after percutaneous atherectomy, all specimens were fixed in 4% paraformaldehyde in PBS.

Subsequently, specimens were processed for paraffin embedding. Sequential sections (4μm 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 lab, 1:45), smooth muscle alpha-actin (Sigma; 1:300), and non-phospho beta-catenin (Cell Signaling; 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 as well as expression of podocan and non-phospho beta-catenin was performed using a computer-assisted morphometry system as described above. Nuclei were counted per area and used to calculate the cell density per mm², podocan expression was
measured as percentage of intimal area covered by podocan staining and expression of non-phospho beta-catenin was measured as percentage of intimal cells with nuclear non-phospho beta-catenin labeling. Ten randomly selected intimal areas, each encompassing 0.04 mm², were assessed per tissue sample as previously described.

Statistical Analysis

SPSS/PC+ software 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-/- and WT genotype. After testing for normal distribution and equality of variances with Levene’s F-test, the independent sample t-test was used to compare intimal SMC density (cells per mm²), podocan expression (percentage of intimal area covered by podocan staining) and expression of the Wnt-TCF pathway marker non-phospho beta-catenin in SMC (percentage of intimal SMCs labeled positive) in primary versus restenotic coronary lesions. Absorption at OD588 (migration assay) and OD490 (proliferation assay) were also compared using the independent sample t-test. Probability values were two-tailed and corrected for ties. P values <0.05 were considered significant.

Results

Expression of Podocan in Injured Mouse Femoral Artery

In non-injured femoral arteries of WT animals podocan expression could not be detected by immunostaining (Fig.1a and d). In contrast, podocan was found consistently in arteries of WT mice after injury. Podocan deposition was seen surrounding medial and neointimal SMCs (Fig.1b and e). Injured arteries of podocan-/- mice were completely devoid of podocan, as
expected, confirming the specificity of the podocan antibody (Fig.1c and f). The complete time course analysis of podocan expression in WT arteries using antibodies for podocan and alpha-actin showed barely detectable podocan staining at 1 week in alpha-actin positive media (Fig.1g and h). At 2 weeks post injury a strong, albeit patchy, podocan expression emerged in the media alongside with strong alpha-actin expression in the media (Fig.1i and j). At 4 weeks most neointimal cells expressed alpha-actin and were surrounded by podocan staining largely of the ECM (Fig.1k 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-/− mice (n=28). At 1 and 2 weeks, no significant difference in neointima size was found between the groups (1 week: 2.0±0.9 vs. 1.8±0.8 x10⁻³ mm², P=NS; 2 weeks: 3.8±1.0 vs. 2.9±0.9 x10⁻³ mm², P=NS) (Fig.2a-f and m). At 4 weeks, however, the neointima area was significantly greater with podocan-/− genotype compared to WT (11.6±1.8 vs. 4.4±1.3 x10⁻³ mm², P<0.05) (Fig.2c, f, m). The neointima to media ratio was also increased with podocan-/− genotype at 4 weeks (3.04±0.44 vs. 1.14±0.15; P<0.01). SMC-density did not show a significant difference between both groups early post injury (1 week: 2078±978 vs. 1958±934 x10³ cells/mm², P=NS; 2 weeks: 8822±2078 vs. 7823±1934 x10³ cells/mm², P=NS) (Fig.2g-l and n). At 4 weeks, however, SMC-density of neointima was significantly increased with podocan-/− genotype (9989±2778 vs. 5813±2012 x10³ cells/mm², P<0.05) (Fig.2n). At 1 week, 4.4±1.0% of cells expressed the proliferation marker Ki-67 with podocan-/− and 4.1±0.8% with WT genotype (P=NS) (Fig.3). At 2 weeks, Ki-67 expression decreased in both groups (2.3±1.1% vs. 2.2±0.9%; P=NS). However, with podocan-/− genotype Ki-67 expression increased again at 4 weeks (7.3±1.9% vs. 2.4±1.0%; P<0.05) (Fig.3). Reendothelialization did not differ between the groups.
(1 week: 27±2% vs. 29±4%, P=NS; 2 weeks: 57±5% vs. 54±4%, P=NS; 4 weeks: 79±4% vs. 84±4%, P=NS). Of note, neointima area (12.8±1.7 vs. 4.6±1.4 x10⁻³ mm², P<0.05) and expression of the proliferation marker Ki-67 (6.0±1.3% vs. 0.0±0.0%, P<0.05) remained significantly increased with podocan-/- genotype even 6 weeks after injury (Figure 4a-f and j). Measuring proliferation by BRDU-incorporation confirmed the increased SMC proliferation found with podocan-/- genotype (18±3% vs. 2±2%, P<0.05) (Fig.4g to k).

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

In WT aortic explants, there was no cellular outgrowth at 3 days in all 8 samples (Fig.5a). In contrast, at the edge of podocan-/- aortic explants, SMC outgrowth was visible in 6 out of 8 samples at 3 days indicating early SMC outgrowth (Fig.5b). Subsequently, we compared migration of the cultured SMCs and found that podocan-/- SMCs grown in 10% FBS migrated significantly faster than WT cells (0.73±0.06 vs. 0.55±0.03, P<0.05) (Fig.5c). Podocan-/- SMCs also grew at a significantly greater rate than WT cells when cultured in 10% FBS or in response to recombinant PDGF (10ng/ml) (10% FBS: 0.76±0.03 vs. 0.69±0.03, P<0.05; 10ng/ml PDGF: 1.01±0.03 vs. 0.89±0.03, P<0.05) as measured by the MTS assay (Fig.5d). In an attempt to restore the WT SMC phenotype we transfected podo-/- SMC with podocan expressing vector. Podocan synthesis was induced in podocan-/- SMCs treated with podocan expressing vector as confirmed by Western Blot (data not shown). Proliferation in both 10% FBS (0.35±0.01 vs. 0.40±0.01, P<0.05) and with PDGF stimulation (0.49±0.02 vs. 0.65±0.02, P<0.05) was significantly reduced as compared to empty vector treatment and approached that seen with WT cells (Fig.5e). Moreover, in human SMC, treated with human podocan expressing vector, podocan was highly enriched compared to vector control and untreated SMC as assessed by Western Blot (Fig.5i). Podocan overexpression resulted in a 29% reduction of SMC migration.
(0.40±0.08 vs. 0.56±0.09, P<0.05)(Fig.5j). Using a BRDU incorporating assay we also found a
time-dependent inhibition of SMC proliferation up to 32% (at 24h) with podocan overexpression
(0.15±0.01 vs. 0.22±0.01, P<0.05 at 24 hours)(Fig. 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 SMC

We found a reduction in phosphorylated and an increase in non-phosphorylated beta-catenin in
podocan-/- SMC compared with WT indicative of Wnt-pathway activation (Fig.5f). To confirm
the increase in transcriptional Wnt-activity in podocan-/- SMCs, we performed

TOPflash/FOPflash reporter assays. The TOPflash/FOPflash assays showed greater than 2-fold
enhancement in nuclear beta-catenin/Tcf/Lef-1 transcriptional activity in podocan-/- SMCs
confirming activation of Wnt-signaling (Fig.5g). When we treated podocan-/- SMCs with beta-
catenin small inhibitory (si)RNA, we observed a significant suppression of non-phosphorylated
beta-catenin compared to control siRNA treatment (data not shown). Of note, beta-catenin RNA
silencing resulted in inhibition of SMC proliferation comparable to the inhibition achieved by
WT podocan gene transfection into podocan-/- SMC (Fig.5h). Of note, the in vivo expression of
non-phosphorylated (stable) beta-catenin was also strongly increased in podocan-/- neointima
compared to WT (2weeks: 10±3% vs. 4±2%, P>0.05; 4weeks: 38±8% vs. 8±3%, P<0.05)
(Fig.6a-f and l). Importantly podocan-/- SMC in the neointima displayed nuclear non-phospho
beta-catenin staining indicative of nuclear beta-catenin translocation, a hallmark of Wnt-
activation (Fig. 6g-i). Conversely, enriching the human form of podocan in human SMC by
treatment with podocan expressing vector resulted in a significant increase in phosphorylated
beta-catenin over non-phosphorylated beta-catenin seen by Western Blot (Fig.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 (Fig.7). In restenotic lesions podocan expression was significantly decreased with 8±2% of intimal area compared with 30±4% in primary coronary lesions (P<0.05). In contrast, intimal cell density was significantly increased in restenotic compared to primary coronary lesions with 632±107 versus 195±40 cells per mm² (P<0.05). Of note, the expression of non-phospho beta-catenin was strongly increased in restenotic lesions compared to primary lesions (22±5% vs. 5±1%, P<0.05). Importantly, nuclear staining of non-phospho beta-catenin indicative of nuclear translocation was observed in restenotic lesions (Fig.8).

Immunofluorescence labeling showed a co-localization of non-phospho beta-catenin and smooth-muscle alpha-actin in hyperplastic areas of restenotic lesions (Fig.8). In all coronary lesions we observed an inverse correlation between the extent of podocan deposition and non-phospho beta-catenin expression (r=-0.78, P<0.05) and a strong positive correlation between the expression of non-phospho beta-catenin and intimal cell density (r=0.94, P<0.05). Of note, in both lesion types staining with an isotype control antibody that matches the podocan antibody did not show any staining excluding non-specific labeling or autofluorescence artifact (Fig.7).

Discussion

Our results suggest that the novel SLRP podocan is a key regulator of the SMC response after arterial injury. Lack of podocan expression with podocan-/- genotype resulted in late and prolonged SMC proliferation after arterial injury yielding exuberant arterial lesion formation. Arterial response to injury critically involves the migration and proliferation of SMC from the media into the intimal space with subsequent ECM synthesis and remodeling events.31-33
showed in our study that podocan is selectively enriched in the ECM of arteries post injury in vivo and we demonstrated in vitro, that podocan is capable of inhibiting SMC proliferation and migration. Since the inception of mechanical treatments for human atherosclerosis beginning with balloon angioplasty and later forms of percutaneous coronary interventions such as stenting the control of the SMC response has remained the Achilles heel of this approach. While stents have vastly improved upon the recoil and constrictive remodeling component of restenosis the underlying problem of accelerated intimal SMC growth has remained. A myriad of strategies and an enormous research effort over many years to control the migratory and proliferative response of SMC post PCI have not resulted in a perfect solution for this problem yet. The current approach of delivering stents releasing non-specific agents promoting cell death and/or inhibition of proliferation has evolved in experimental models and multiple clinical trials and has been successful at lowering the need for recurrent vascular interventions. However, this success comes at the expense of delaying vascular healing, whose ultimate long-term clinical impact is still being evaluated and debated. Irrespective of this ongoing debate it remains obvious from a vascular biology point of view that the current agents released by stents to control the SMC proliferative and migratory response are by no means physiologic inhibitors of this process. Rather, they are pretty blunt instruments imparting short- and long-term negative effects on the healing arterial wall such as delayed re-endothelialization, increased inflammation and enhanced thrombogenicity.

The possible role of podocan as physiologic inhibitor of the SMC migratory and proliferative response is suggested by several observations. Podocan is not expressed constitutively at high levels by vascular SMC at baseline. In the absence of injury podocan expression could not be detected by imunohistochemistry. Only after arterial injury podocan was
gradually expressed at increasing and robust levels in the media and neointima between 2 and 4 weeks after injury. Of note, in podocan-/- mice arterial lesion formation was affected specifically between 2 and 4 weeks after injury compared with WT. This is precisely the time when ECM synthesis and podocan deposition typically takes 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.\(^{33,40,41}\) Consistent with the postulated inhibitory regulatory function of podocan, we found an increase in proliferation in neointima devoid of podocan in animals with podocan-/- genotype as late as 4 weeks after injury. This finding is very unusual because the natural history of arterial wall cell proliferation in most models peaks during the first two weeks and tapers off at later time points.\(^{33,41}\) In addition to the late increase in proliferation, we also found a late increase in SMC-density at 4 weeks in the podocan-/- neointima. Previous studies reported that by 4 weeks SMC density usually decreases due to a decreased rate of SMC proliferation and ongoing ECM synthesis.\(^{33,41}\) Even as late as 6 weeks after arterial injury robust SMC proliferation was still evident in the neointima of podocan-/- mice.

To extend these in vivo observations and to better define the podocan genotype effect, we explanted SMCs from podocan-/- and WT aortas.\(^{42}\) The podocan genotype determined the rate of migration and proliferation of the cultured SMCs with strongly enhanced migration and proliferation of SMC lacking podocan expression. Transfection of podocan-/- SMC with WT podocan decreased proliferation to WT levels, essentially “normalizing” the podocan-/- phenotype and indicating a specific podocan effect. To test if podocan has a true inhibitory effect also on podocan competent WT cells and to assess the function of the human isoform of podocan we overexpressed human podocan in human SMC. Consistent with our observations in murine
SMC, transfection of SMC with the human form of podocan significantly reduced migration and proliferation.

To define the possible mechanism through which podocan exerts its effect on SMC 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 two different experimental settings. Cultured murine SMCs with podocan-/- genotype showed increased Wnt-TCF pathway activation compared to WT SMC. Conversely, human podocan competent SMC’s overexpressing podocan showed a significantly decreased Wnt-TCF pathway activation. These corresponding changes were verified using two independent and established methods of measuring Wnt-TCF-pathway activation (the relevant methodology is further outlined in the supplemental method section).

Further evidence for an important role of the Wnt-TCF pathway in mediating podocan-related effects on SMC also stems from the observation, that beta-catenin siRNA treatment was capable of normalizing increased podocan-/- SMC proliferation in a similar fashion as the restoration of podocan expression with podocan transfection did.

Going back to the in vivo physiology of the arterial response to injury we wanted to test if matching alterations in the Wnt-TCF pathway could also be found in the neointima with podocan-/- and WT genotype. Interestingly, we observed a strong increase in the expression of the non-phosphorylated form of beta-catenin in the neointima with podocan-/- genotype. Podocan-/- neointimal SMC exhibited a nuclear staining pattern suggestive of nuclear translocation of beta-catenin and hence indicative of Wnt-TCF-pathway activation. The peak of this Wnt-TCF-pathway activation in neointimal SMC coincided precisely with the late rise in SMC proliferation in podocan-/- neointima. As final step we set out to determine the relevance of
podocan and its effects on the Wnt-TCF pathway for the patho-physiology 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 activation, whom we found elevated in podocan-/- neointima. Podocan deposition in restenotic coronary lesions was significantly decreased compared with primary lesions whereas the expression of non-phospho beta-catenin was significantly increased in human coronary restenotic tissue yielding a significant inverse correlation. This finding is intriguing since human restenotic tissue is characterized by some of the same features observed in podocan-/- neointima. A steep increase in cell density, increased migration and increased proliferative events, all features, which are typical of tissues with elevated levels of Wnt-TCF pathway activation as has been extensively described in cancer literature. This first report of the inverse relationship between podocan expression and the expression of Wnt-TCF pathway related molecules in human vascular lesions provides further evidence for a possible role of podocan as physiologic inhibitor of the SMC migratory and proliferative response in the arterial response to PCI in patients. Its effects on SMC 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.

Acknowledgments: We thank David J. Schneider, MD, for his expert review of the manuscript and we thank Renata Hutter, MA, for expert help in data acquisition and analysis.

Funding Sources: This work was supported in part by grant DK 56492 (to PEK) and by NIH-training grant 5 T32 HL 7824-13 (to RH).
Conflict of Interest Disclosures: None.

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Table 1. Coronary Atherectomy Samples: Patient and Lesion Characteristics

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<th>Cells/mm²</th>
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Figure Legends:

Figure 1. Effect of injury and podocan genotype on arterial podocan expression. (a and d): Podocan is not detected in non-injured wild type femoral arteries, x200, x1000; scale bar=50 μm. (b and e): After injury brown labeling (arrows) for podocan is clearly seen in media (me) and neointima (ni) of wild type arteries at 4 weeks; x200, x1000; scale bar=50 μm. (c and f): After injury an artery with podocan-/- genotype exhibits a large neointima (ni) and shows, as expected, no podocan labeling serving as negative control for podocan immunostaining; x200, x1000; scale bar=50 μm. Time course of podocan expression post injury in wild type artery: 1 week (g-h): At
1 week after injury podocan is hardly detectable in the media (me) while alpha-actin is expressed strongly. x200; scale bar=50 μm. 2 weeks (i-j): Much stronger, albeit patchy, medial (me) podocan expression appears (arrows). Of note, podocan is also seen in early neointima (ni), x200; scale bar=50 μm. 4 weeks (k-l): Podocan expression (arrows) remains strong in neointima and media in close spatial association with alpha-actin signals, x200; scale bar=50 μm.

**Figure 2.** Time course of arterial response to injury with wild type and podocan-/- genotype. Combined Masson Elastin staining: WT genotype (a-c), podocan-/- genotype (d-f): 1 week (a and d): Cells adhere along the arterial surface on the luminal side of the media (me) and an adventitial cellular infiltrate forms in both groups in a similar fashion; x200; scale bar=50 μm. 2 weeks (b and e): A comparably sized typical early neointimal lesion (ni) with densely packed cells has formed in both groups; x200; scale bar=50 μm. 4 weeks (c and f): A moderately sized arterial lesion (ni) has formed with WT genotype; in contrast, with podocan-/- genotype the neointima shows a strongly increased size; x200; scale bar=50 μm. Bar graph (m): Comparison of neointima formation with WT and podocan-/- genotype: neointima area in x10^{-2} mm^2 (independent sample t-test). Smooth Muscle Alpha-Actin: WT genotype (g-i), podocan-/- genotype (j-l): 1 week (g and j): Alpha-actin expression (brown labeling) is predominantly seen in the media (me) in both groups, no neointima has formed yet; x200. 2 weeks (h and k): Nascent neointimal (ni) alpha-actin expression and a trend towards higher SMC numbers with the podocan-/- genotype can be seen; x200; scale bar=50 μm. 4 weeks (i and l): A steep increase in the numbers of alpha-actin positive cells can be observed in neointima (ni) with podocan-/- genotype; x200; scale bar=50 μm. Bar graph (n): Comparison of neointimal SMC density with WT and podocan-/- genotype: cell density in x10^3 mm^2 (independent sample t-test).
Figure 3. Arterial proliferation with wild type and podocan-/− genotype. Ki-67 (FITC) and smooth muscle alpha-actin (Texas Red) double labeling: WT genotype (a-f): podocan-/− genotype (g-l): 1 week (a and g): Early after injury Ki-67 positive (green) and alpha-actin positive (red) SMC (arrow) are seen in the media (me) in both groups; x200; scale bar=50 μm. (d and j represent matching IgG-isotype control stainings). 2 weeks (b and h): At this time only few Ki-67 signals (arrows) are seen in both groups consistent with a gradual decline in proliferation after the first week; x200; scale bar=50 μm. (e and k negative controls). 4 weeks (c and i): An unusually late rise in proliferation of SMC (red alpha-actin labeling) is detected by nuclear Ki-67 (green) labeling (arrows) with podocan-/− genotype; x200; scale bar=50 μm. (f and l negative controls). Bar graph (m): Comparison of Ki-67 expression with WT and podocan-/− genotype after arterial injury: expression in % cells (independent sample t-test).

Figure 4. Late arterial proliferation with wild type and podocan-/− genotype. WT genotype (a, d, g): podocan-/− genotype (b, e, h): H&E staining (a-c): Compared with WT (a) the strong increase in neointima (ni) with podocan-/− genotype (b) persists even at 6 weeks. Non-injured artery (c) of podocan-/− mouse serves as negative control for proliferation labeling; x200; scale bar=50 μm. Ki-67 and smooth muscle alpha-actin double labeling (d-f): At 6 weeks no green (FITC) Ki-67 labeling is seen in neointima (ni) and media (me) with WT genotype (d). With podocan-/− genotype, however, green nuclear Ki-67 labeling (arrows) is seen in neointima (ni) even 6 weeks after injury (e). Non-injured podocan-/− contra-lateral artery does not show any ki-67 signals (f). x200; scale bar=50 μm. BRDU-Labeling (g-i and k): In smaller WT neointima (ni) BRDU-labeling is absent at 6 weeks after injury (g). In contrast, multiple nuclei with brown labeling (arrows) indicating BRDU-incorporation are seen in hypercellular neointima with podocan-/−.
genotype (h); x200; scale bar=50 μm. Brown BRDU labeling (arrows) is also seen in bone marrow (bm) and serves as positive control (i). Brown labeling is absent with isotype-control staining in bone marrow; x400; scale bar=50 μm (k). Bar graph (j): Comparison of Ki-67 and BRDU expression with WT and podocan-/- genotype 6 weeks after arterial injury: expression in % cells (independent sample t-test).

**Figure 5.** Effect of WT and podocan-/- genotype on murine aortic SMC: Aortic explant culture (a and b): (a) The edge of WT aortic explant shows no SMC outgrowth at day 3; x400; scale bar=50 μm. (b) In contrast, numerous SMCs are seen at the edge of a podocan-/- aortic explant at the same time point; x400; scale bar=50 μm. SMC migration and proliferation (c-e): (c) SMC migration is increased with podocan -/- genotype compared with WT in a colorimetric test based on the Boyden chamber principle (independent sample t-test). (d) Podocan-/- SMCs also grow faster as measured by the MTS assay (independent sample t-test). (e) Podocan-/- SMC transfected with podocan vector slow their growth to WT-level (independent sample t test). Wnt-TCF pathway activation (f-h): (f) In SMC with podocan-/- genotype the ratio of phosphorylated to non-phosphorylated beta-catenin is reversed as seen by Western blot. (g) Transcriptional activity of Wnt-TCF pathway measured directly by TOPflash/FOPflash assay is also increased with podocan-/- genotype. (h) Podocan-/- SMCs treated with small inhibitory RNAs to beta-catenin show inhibition of growth. Effect of podocan overexpression on human aortic SMC: Western Blot (i): Western Blot confirms overexpression of the human form of podocan. Podocan in control and empty vector treated SMC is below the detection threshold. Migration (j): SMC migration is reduced by 29% with podocan overexpression. Proliferation (k): SMC proliferation is reduced by 32% with podocan overexpression. Wnt-TCF pathway
activation (l): SMC overexpressing podocan show an increase in phosphorylated-beta catenin on Western Blot compared to non-treated or empty vector treated SMC indicating Wnt-TCF pathway suppression.

**Figure 6.** Effect of wild type and podocan-/- genotype on Wnt-pathway after arterial injury:

Alpha-actin (Texas-Red) and non-phospho beta-catenin (FITC) double-labeling: WT genotype (a and d), podocan-/- genotype (b-c and e-f, and g-k): Low power magnification (a–f): An antibody specific for the non-phosphorylated form of beta-catenin gives much stronger green (FITC) signals in neointima (ni) with podocan -/- genotype (b and e) compared with WT (a and d); x200; scale bar=50 μm. Matching isotype controls are shown for podocan-/- neointima (c and f). High power magnification (g–k): Comparing single DAPI (g), single non-phospho-beta-catenin labeling (h) and both combined (i) under high power magnification demonstrates clearly the nuclear location of beta-catenin signals in podocan-/- neointima indicating beta-catenin nuclear translocation – a hallmark of true Wnt-pathway activation; x1000; scale bar=50 μm. These cells also stain positive for alpha-actin (j) identifying them as SMC and show co-localization with non-phospho beta-catenin signals (k). Bar graph (l): Comparison of non-phospho beta-catenin expression in neointima with WT and podocan -/- genotype after arterial injury: expression in % cells (independent sample t-test).

**Figure 7.** Expression of Podocan in Human Atheroma: Primary Carotid Atheroma (a to d), Primary Coronary Lesion (e-f and i-j, and m-n, q-r, u-v), Restenotic Coronary Lesion (g-h and k-l, and o-p, s-t, w-x): Podocan immunostaining (a–d): An antibody specific for the human form of podocan gives strong brown labeling in the intima of carotid atheroma (a and c); x100; scale
Combined Masson Elastin (CME) staining (e–g): Comparing the histo-architecture of primary and restenotic coronary lesions shows distinct differences. Spindle shaped SMC are surrounded by large spaces of ECM at a rather low cell density in primary lesions (e). In restenotic tissue abundant numbers of SMC are tightly clustered and surrounded by a comparatively smaller ECM space (g); x50, x100, x200; scale bar=50 μm. Two Versions of Podocan and Smooth Muscle Cell Double Labeling (f–v): Smooth muscle alpha-actin (FITC) and podocan (Texas-Red) double-labeling (f, h): Low power magnification images reveal 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. Smooth muscle alpha-actin (Texas-Red) and podocan (FITC) double-labeling (j, m, n and r, u, v): With reversed double labeling higher power magnification images confirm that large ECM spaces in primary lesions surrounding the red-labeled SMC are enriched with podocan as shown by extensive green (arrows) labeling (j, m, n and r, u, v). In contrast, green podocan labeling (arrows) in restenotic tissue covers a much smaller area and is restricted to the immediate vicinity of red-labeled SMC (l, o, p and t, w, x); x200, x1000; scale bar=50 μm. Corresponding intimal locations in adjacent serial sections are also shown by light microscopy and Combined Masson Elastin (CME) staining (i, q and k, s); x200, x1000; scale bar=50 μm. Of note, in both lesion types staining with an isotype control antibody that matches the podocan antibody does not show any green signals (z, I-IV). Bar graph (y): Comparison of podocan expression (% area) and SMC density (cells per mm²) in primary and restenotic coronary lesions: (independent sample t-test).

**Figure 8.** Activation of Wnt-TCF Pathway in Human Atheroma: Primary Coronary Lesion (a, c,
e), Restenotic Coronary Lesion (b, d, f, h, i): Alpha-actin (Texas-Red) and non-phospho beta-catenin (FITC) double-labeling: An antibody specific for the non-phosphorylated form of beta-catenin gives strong green (FITC) signals in intimal cells of a restenotic coronary lesion (f), whereas no signals are seen in the intima of a primary lesion (e); x400; scale bar = 50 µm. These cells also have typical morphology of SMC (b) and stain positive for alpha-actin (d); x400; scale bar = 50 µm. High power magnification (h–i): Comparing single non-phospho-beta-catenin labeling (h) and combined labeling with DAPI (i) demonstrates clearly the nuclear location of beta-catenin signals in restenotic intima indicating beta-catenin nuclear translocation – a hallmark of true Wnt-TCF pathway activation; x1000; scale bar = 50 µm. Bar graph (g): Comparison of non-phospho beta-catenin expression in the intima of primary and restenotic coronary lesions: expression in % cells (independent sample t-test).
Effect of Injury and Podocan Genotype on Podocan Expression

No injury
Podocan +/+ 

4 weeks post injury
Podocan +/+ 

4 weeks post injury
Podocan +/-

Time Course of Podocan Expression Post Injury With WT-Genotype

Podocan +/-

1 week post injury
anti-podocan

2 weeks post injury
anti-smooth muscle alpha actin

4 weeks post injury

Figure 1
Figure 2

- Podocan +/+:
  - 1 week post injury: a
  - 2 weeks post injury: b
  - 4 weeks post injury: c

- Podocan -/-:
  - 1 week post injury: d
  - 2 weeks post injury: e
  - 4 weeks post injury: f

- Podocan +/+:
  - 1 week post injury: j
  - 2 weeks post injury: k
  - 4 weeks post injury: l

- Podocan -/-:
  - 1 week post injury: g
  - 2 weeks post injury: h
  - 4 weeks post injury: i

Graphs m and n show:
- m: Neointimal Area
  - x 10^6/mm^2
  - 1 week
  - 2 weeks
  - 4 weeks
  - P < .05
  - P = NS

- n: Neointimal SMC Density
  - x 10^6 cells/mm^2
  - 1 week
  - 2 weeks
  - 4 weeks
  - P = NS
  - P < .01
Figure 4

Podocan +/+ 6 weeks post injury

Podocan +/- 6 weeks post injury

Podocan +/- No injury

Figure 4

% cells +Ki67

Ki67 Proliferation Index

Arterial Wall

% cells +BRDU

BrdU Proliferation Index

Arterial Wall

j

k

Figure 4
Figure 6

Anti-alpha-actin (Texas Red) and non-phospho beta-catenin (FITC)

Podocan +/+ 4 weeks post injury

a) anti-alpha-actin
b) anti-alpha-actin

Podocan -/- 4 weeks post injury
c) isotype control
d) anti-NPBC
e) anti-NPBC

High Power Magnification in Podocan -/- Neointima

j) DAPI + anti-alpha-actin
k) overlay
l) DAPI + anti-NPBC

% Cells

Beta-Catenin Expression

P = NS

P < .05

2 weeks 4 weeks
Figure 8
The 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
Randolph Hutter, Li Huang, Walter S. Speidl, Chiara Giannarelli, Paul Trubin, Gerhard Bauriedel, Mary E. Klotman, Valentin Fuster, Juan J. Badimon and Paul E. Klotman

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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.