Phenotypic modulation of smooth muscle cells in atherosclerosis is associated with downregulation of \textit{LMOD1, SYNPO2, PDLIM7, PLN} and \textit{SYNM}.

Matic Perisic L\textsuperscript{1}, Rykaczewska U\textsuperscript{1}, Razuvaev A\textsuperscript{1}, Sabater-Lleal M\textsuperscript{2}, Lengquist M\textsuperscript{1}, Miller CL\textsuperscript{3}, Ericsson I\textsuperscript{1}, Röhl S\textsuperscript{1}, Kronqvist M\textsuperscript{1}, Aldi S\textsuperscript{1}, Magné J\textsuperscript{2}, Paloschi V\textsuperscript{2}, Vesterlund M\textsuperscript{6}, Li Y\textsuperscript{2}, Jin H\textsuperscript{2}, Gonzalez Diez M\textsuperscript{2}, Roy J\textsuperscript{2}, Baldassarre D\textsuperscript{5,6}, Veglia F\textsuperscript{6}, Humphries SE\textsuperscript{7}, de Faire U\textsuperscript{8,9}, Tremoli E\textsuperscript{5,6}, on behalf of the IMPROVE study group, Odeberg J\textsuperscript{10}, Vukojević V\textsuperscript{11}, Lehtiö J\textsuperscript{4}, Maegdefessel L\textsuperscript{2}, Ehrenborg E\textsuperscript{2}, Paulsson-Berne G\textsuperscript{2}, Hansson GK\textsuperscript{2}, Lindeman JHN\textsuperscript{12}, Eriksson P\textsuperscript{2}, Quertermous T\textsuperscript{3}, Hamsten A\textsuperscript{2}, Hedin U\textsuperscript{1}.

\textsuperscript{1}Department of Molecular Medicine and Surgery, Karolinska Institutet, Sweden, \textsuperscript{2}Department of Medicine, Karolinska Institutet, Sweden, \textsuperscript{3}Division of Vascular Surgery, Stanford University, USA, \textsuperscript{4}Science for Life Laboratory, Sweden, \textsuperscript{5}Dipartimento di Scienze Farmacologiche e Biomolecolari, Università di Milano, Milan, Italy, \textsuperscript{6}Centro Cardiologico Monzino, IRCCS, Milan, Italy, \textsuperscript{7}British Heart Foundation Laboratories, University College of London, Department of Medicine, Rayne Building, London, United Kingdom, \textsuperscript{8}Division of Cardiovascular Epidemiology, Institute of Environmental Medicine, Karolinska Institutet, \textsuperscript{9}Department of Cardiology, Karolinska University Hospital Solna, Karolinska Institutet, Stockholm, Sweden, \textsuperscript{10}Science for Life Laboratory, Department of Proteomics, Sweden, \textsuperscript{11}Department of Clinical Neuroscience, Center for Molecular Medicine, Karolinska Institutet, Sweden, \textsuperscript{12}Department of Vascular Surgery, Leiden University Medical Center, Netherlands.

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Correspondence to:

Ljubica Perisic Matic

Department of Molecular Medicine and Surgery, Solna

Karolinska Institute, L8:03

SE-171 76 STOCKHOLM

Tel: +46-76-0237008, E-mail: Ljubica.Perisic@ki.se
Abstract

Objective: Key augmented processes in atherosclerosis have been identified, whereas less is known about downregulated pathways. Here we applied a systems biology approach to examine suppressed molecular signatures, with the hypothesis that they may provide insight into mechanisms contributing to plaque stability.

Approach and Results: ‘Muscle contraction’, ‘muscle development’ and ‘actin cytoskeleton’ were the most downregulated pathways (FDR=6.99e-21, 1.66e-6, 2.54e-10 respectively) in microarrays from human carotid plaques (n=177) vs. healthy arteries (n=15). In addition to typical SMC markers, these pathways also encompassed cytoskeleton-related genes previously not associated with atherosclerosis. SYNPO2, SYNM, LMOD1, PDLIM7, and PLN expression positively correlated to typical SMC markers in plaques (Pearson r>0.6, p<0.0001) and in rat intimal hyperplasia (r>0.8, p<0.0001). By immunohistochemistry, the proteins were expressed in SMCs in normal vessels, but largely absent in human plaques and intimal hyperplasia. Subcellularly, most proteins localised to the cytoskeleton in cultured SMCs and were regulated by active enhancer histone modification H3K27ac by ChIP-seq. Functionally, the genes were downregulated by PDGFB and IFNg, exposure to shear flow stress and oxLDL loading. Genetic variants in PDLIM7, PLN and SYNPO2 loci associated with progression of carotid intima-media thickness in high-risk subjects without symptoms of cardiovascular disease (n=3378). By eQTL, rs11746443 also associated with PDLIM7 expression in plaques. Mechanistically, silencing of PDLIM7 in vitro led to downregulation of SMC markers and disruption of the actin cytoskeleton, decreased cell spreading and increased proliferation.

Conclusions: We identified a panel of genes that reflect the altered phenotype of SMCs in vascular disease and could be early sensitive markers of SMC dedifferentiation.
Abbreviations

AF- amaurosis fugax
AHA- American Heart Association
AS-asymptomatic
BiKE- Biobank of Karolinska Endarterectomies
CEA- carotid endarterectomy
CP- carotid plaque
CHip- chromatin immunoprecipitation
CNN- calponin
cIMT- carotid intima-media thickness
ECM- extracellular matrix
LMOD1-leiomodin 1
GEO- Gene Expression Omnibus
IHC- immunohistochemistry
IFL- immunofluorescence
MS- minor stroke
MYOC- myocardin
MYH11- myosin heavy chain 11
NA- normal artery
NCA- normal carotid artery
PCNA- proliferating cell nuclear antigen
PDGF- platelet derived growth factor
PLN- phospholamban
PDLIM7- PDZ and LIM domain containing 7
RNAseq- RNA sequencing
qPCR- quantitative polymerase chain reaction
FDR- false discovery rate
S- symptomatic
SMC- smooth muscle cell
SMA- smooth muscle actin
SYNM- synemin
SYNPO2- synaptopodin 2
TIA- transitory ischemic attack
TAGLN- transgelin
Introduction

Unstable atherosclerosis in the carotid bifurcation is a common cause of stroke, and guidelines recommend treatment with stroke-preventive carotid endarterectomy (CEA) in patients with signs of cerebral embolism. Stable, asymptomatic (AS) carotid lesions are generally rich in extracellular matrix (ECM) and smooth muscle cells (SMCs), whereas unstable (symptomatic, S) plaques contain abundant inflammatory cells and a thin fibrous cap prone to rupture. Inflammation, cytokines, mitogens, ECM degradation and altered cell-matrix interactions have been associated with intraplaque processes in atherogenesis which promote activation of contractile SMCs in the media into a secretory and replicating phenotype that engage in intimal remodeling and formation of the fibrous cap.

Contractile SMCs are distinguished from other cell types by expression of a unique repertoire of markers including Smooth Muscle Actin (SMA, ACTA2), Calponin (CNN1), Transgelin (TAGLN), Myocardin (MYOCD) and Myosin Heavy Chain 11 (MYH11), mostly associated with the acto-myosin cytoskeleton. These genes are downregulated in activated SMCs and may be undetectable using traditional immunohistochemical staining methods. However, it is unclear whether altered expression of these genes takes place concomitantly or successively during phenotypic modulation. This problem is notable in atherosclerotic lesions where SMA positive (SMA+) cells define several distinct regions and can be found in the necrotic core and the fibrous cap (Figure 1A). Recently, SMC transdifferentiation into CD68+ macrophage-like cells has been demonstrated in atherosclerosis which further emphasizes the complexity in characterizing SMC phenotypes in vascular disease. Apart from atherogenesis, activation of SMCs also dominates in healing reactions aimed to repair the vessel after injury, healing of ruptured atheromas, restenosis after arterial interventions and in the failure of vein grafts and dialysis fistulas.

Understanding the molecular and cellular processes that convert asymptomatic plaques into symptomatic ones may facilitate the development of preventive pharmacotherapy with unprecedented impact on cardiovascular mortality and morbidity. For this purpose, intensive efforts have been dedicated to the identification of suitable targets through analysis of augmented pathways and molecules in vulnerable lesions. In contrast, less is known about pathways that are inhibited in atherogenesis and in the process of plaque instability. Since identification of such inherent functional changes within the vessel wall may give clues to therapies that can sustain arterial resistance to atherogenic stimuli or improve stability of established complex lesions, studies of downregulated genes and suppressed pathways may be equally important. Identification of ultimately translatable target molecules is bound to be more successful when generated directly from human disease, followed by clinical and experimental exploration.

Recently we performed a comprehensive transcriptomic analysis of late-stage human carotid atherosclerosis based on defined clinical patient phenotypes. Our findings confirmed a central role for lipid accumulation, inflammation and proteases in plaque instability, and highlighted haemoglobin metabolism and bone resorption as important enriched pathways in plaques. Here, we instead analysed downregulated molecular signatures in carotid plaques by applying an integrative framework based on collaboration among three large human biobanks: initial discoveries were made using material from the Biobank of Karolinska Endarterectomies (BiKE, n=177 plaques from end-stage atherosclerosis patients and n=15 macroscopically healthy, normal arteries); data was further validated using atheroprogression samples from the SOKRATES biobank (n=28 patients tissues); and genetic analyses were performed in the IMPROVE cohort (n=3378 high-risk patients without symptoms or signs of cardiovascular disease). We found that SMC-related functional categories were the most significantly affected in plaques and identified a set of downregulated SMC-related genes previously poorly studied in vascular disease. Temporal changes in the expression of these genes were followed in the rat carotid injury model and in primary SMCs in vitro in comparison with typical SMC markers. Genetic association with progression of carotid...
intima-media thickness (cIMT) as a surrogate marker of atherosclerosis, was investigated in the large cohort of high-risk subjects and mechanistic studies were performed for one of these genes \textit{in vitro}. We report a panel of novel SMC markers that are suppressed in vascular disease in humans and may reflect the altered phenotype of SMCs during vascular remodelling.
Material and Methods

Material and Methods are available in the online-only Data Supplement.

Results

Genes and pathways associated with SMCs are repressed in atherosclerosis

Pathways associated with ‘muscle contraction’, ‘muscle development’ and ‘actin cytoskeleton’ were the most significantly downregulated in microarrays comparing late-stage human carotid plaques (CP) vs. normal arteries (NA) as well as in plaques extracted from symptomatic vs. asymptomatic patients (e.g. in CP vs. NA comparison FDR=6.99e-21, 1.66e-6 and 2.54e-10 respectively, Supplementary Table I). Genes clustered in these categories were the typical markers of SMCs and acto-myosin cytoskeleton (Supplementary Fig IA). Among the most significantly downregulated genes appeared several whose function in SMCs was previously unexplored in the context of atherosclerosis: LMOD1 (Leiomodin 1), SYNPO2 (Synaptopodin 2), PLN (Phospholamban), PDLIM7 (PDZ and LiM domain containing 7) and SYNM (Synemin). By constructing functional networks from their extended protein-protein interactions and expression profiles across tissues 14-16, we noted that these genes were co-expressed with actin and microtubule markers (Figure 1B) and some of them also co-interacted with the cytoskeleton based on available public data (i.e. PDLIM7, SYNM, LMOD1 and SYNPO2; Supplementary Figure IB). Strong downregulation of these transcripts was found in two non-overlapping microarray datasets comparing carotid plaques to normal arteries (n=127 CP vs. n=10 NA and n=50 CP vs. n=5 NA, p<0.0001 for most transcripts) and downregulation was also noted in plaques from symptomatic vs. asymptomatic patients (n=87 S vs 40 AS, p<0.01) (Figure 1C, full list in Supplementary Table II). Moreover, the protein levels were also lower in plaques from S vs. AS patients, as determined by mass spectrometry (n=9 S vs 9 AS, Supplementary Figure II). Additionally, a trend towards downregulation of these genes was observed in publicly available microarray datasets comparing human carotid plaques (n=12) vs. normal arteries (n=9)10 and carotid plaques (n=32) vs. matched adjacent tissue (GSE43292). Strong positive correlations were seen between mRNA expression of LMOD1, SYNPO2, PLN, PDLIM7 and SYNM in carotid plaques and typical SMC markers such as ACTA2, MYH11, CNN1, MYOCD (Pearson r>0.6, p<0.0001, representative examples in Figure 1D, full data in Supplementary Table III). Similarly, strong correlations between corresponding protein levels, as determined by mass spectrometry, were also demonstrated (Pearson r>0.8, p<0.0001, Supplementary Table III).

To further investigate the association of the selected genes with SMCs, we analyzed a publicly available microarray dataset (GSE23303) comparing microdissected SMC-rich subintimal regions of carotid plaques with macrophage-rich regions from the necrotic core. We found that mRNA levels of these genes were strongly downregulated in macrophage-rich compared with SMC-rich regions, while no significant difference was seen in this comparison for ACTA2 (Supplementary Table II).

To experimentally corroborate our findings from human plaques, we analyzed the expression of the selected genes in an inducible plaque rupture model on ApoE background, where mice present atherothombotic events and morphological signs of plaque instability 17. Expression of all 5 genes of interest was strongly downregulated in ligated vs. non-ligated arteries (i.e. mRNA mean fold change= -114, p<0.0001 for Synpo2; fold change= -45, p<0.0001 for Lmod1), and marginally also in comparison between ruptured vs. stable plaques, thus replicating results from the human datasets (Supplementary Table IV).

Collectively, these results demonstrated that we have identified a set of previously poorly characterised genes through transcriptomic profiling of late-stage human atherosclerosis, likely associated with loss of contractile SMC features in the disease.
LMOD1, SYNPO2, PDLIM7, SYNM and PLN are expressed by SMCs

The localisation of selected genes and proteins in normal human vessels and carotid plaques was performed by in situ RNA hybridization and immunohistochemistry and compared with typical SMC markers such as MYH11, CNN1 and SMA, including proliferating cell nuclear antigen (PCNA) as a marker of cell proliferation. By immunohistochemistry, MYH11, considered to be an early marker of phenotypic changes in SMCs\(^3\), was only detected in PCNA- SMCs in normal carotid artery media but was absent in late-stage plaques, while CNN1 was detectable in the normal artery as well as in subintimal SMA+/PCNA- cells and in SMA+ cells in the fibrous cap of carotid plaques (Figure 2A). By co-immunostaining, SMA, LMOD1, SYNPO2, PDLIM7, SYNM and PLN were all abundantly expressed in SMCs in normal arteries (carotid artery stainings shown in Figure 2B). The stainings appeared mostly cytosolic, except in the case of PLN, which exhibited nuclear staining. In late-stage plaques, PDLIM7 was present in subintimal SMCs and weak expression was also detected in stellate-shaped SMA+ cells in the fibrous cap. SYNM showed a similar staining pattern in subintimal SMCs but was absent from the fibrous cap. SYNPO2, LMOD1 and PLN were not detectable in these plaques by immunohistochemistry. RNA transcripts of MYH11, CNN1, ACTA2 and selected genes were all detectable in the normal artery media and to a lesser extent also in cells with elongated nuclei in the fibrous cap (except PLN, Supplementary Figure III).

We further investigated the expression of these genes during atheroprogression, using human aortic lesions from different stages of disease graded according to the modified American Heart Association criteria\(^18\), ranging from adaptive intimal thickening and xanthomas (stages I and II), via pathological intimal thickening (stage III) to early and thin-cap fibroatheromas (stages IV and V). In these lesions, SMA and CNN1 were detectable in SMCs from early stages to advanced lesions, while MYH11 as expected, was absent from PCNA+ SMCs already at stage I (Supplementary Figure IV). LMOD1 and SYNPO2 were mostly absent already from stage I, PLN was not detectable from stage III, whereas SYNM and PDLIM7 were present in subintimal SMCs but sparsely in cells that build the fibrous cap from stage III. Interestingly, in human intimal hyperplasia, we observed the reappearance of both typical SMC markers and the selected genes in SMA+/PCNA- areas (Figure 2).

Abundant signal was observed for CNN1 as well as for PDLIM7 and SYNM, while LMOD1, SYNPO2 and PLN were sparsely present. Our results confirm that these genes localise to quiescent SMCs in normal artery media and undergo various degrees of downregulation at both transcript and protein levels in activated SMA+ cells of lesions, with reappearance in mature intimal hyperplasia. Of note, these proteins were also detected in SMA+ cells in several other smooth muscle-rich tissues (Supplementary Figure V).

*Lmod1, Synpo2, Pdlim7, Synm* and *Pln* are downregulated early in response to experimental vascular injury but reappear in mature neointima

Time-dependent alterations in expression of SMC markers were examined by transcriptomic analysis from rat carotid arteries after balloon injury (Figure 3). Typical SMC genes along with *Lmod1, Synpo2, Pdlim7, Synm* and *Pln* showed similar gene expression profiles with gradual downregulation in the early phases after injury, but upregulation after 2-12 weeks in the mature neointima. Expression correlations of *Lmod1, Synpo2, Pln, Pdlim7* and *Synm* with typical SMC markers in this model were strongly positive (mostly Pearson r>0.8, p<0.0001, Figure 3C, Supplementary Table III) and negative with cytokines such as *Pdgfb, Igf1* and *Tgfb1* (Figure 3C). By IHC, we observed loss of CNN1 from PCNA+ SMC layers closer to the lumen, while the staining was still present in deeper medial layers at day 5 and again abundant in the mature intima with reduced PCNA staining 12 weeks after injury (Figure 4A). Staining for LMOD1, SYNPO2, PDLIM7, SYNM and PLN was absent at day 5 with gradual reappearance in medial SMCs in tissues with pronounced intimal hyperplasia 12 weeks after injury (Figure 4B). No similar changes in gene expression patterns were found in contralateral uninjured arteries (Supplementary Figure VI). These analyses indicated that downregulation of *Lmod1, Synpo2, Pln, Pdlim7* and *Synm* might functionally relate to SMC activation in response to injury.
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**LMOD1, SYNPO2, SYNM, PDLIM7 and PLN localise mostly to actin-cytoskeleton in SMCs and relate to phenotypic changes in vitro**

To address the expression of the selected genes during the process of SMC phenotypic modulation, rat aortic SMCs were isolated by collagenase digestion, seeded on fibronectin and cultured in serum-free medium or medium supplemented with PDGFBB for 7 days. Directly upon isolation (day 0), almost 90% of the cells were SMA+ by flow cytometry, although lower SMA levels in a subgroup of cells were detectable (Figure 5A). After 7 days, 95% of the population cultured in serum-free medium uniformly expressed higher levels of SMA, while cells stimulated with PDGFBB showed presence of two subpopulations of which one expressed lower signal for SMA (totally 77% SMA+ cells, Figure 5A, detailed analysis shown in Supplementary Figure VII). By qPCR, mRNA levels of Acta2, Myocd and Myh11 as well as Lmod1, Synpo2, Pdlim7, Synm and Pln strongly decreased from day 1 to day 3 in culture, but on day 5 and 7 the expression of most of these genes (except Synm) gradually increased. At each reference time-point, cells cultured in the presence of PDGFBB showed downregulation of the target genes compared to cells in serum-free medium (Figure 5B). By RNA-seq, downregulation of LMOD1, SYNPO2, PDLIM7, SYNM and PLN was also observed in low-passage human SMCs cultured in serum-supplemented (de-differentiation condition) vs. serum-free medium (Supplementary Table V).

By ChIP-seq, we observed that all these genes were under the regulation of active enhancer histone modification H3K27ac (Supplementary Table VI). Prediction of putative binding motifs in genomic sequences using MSigDB software searching a span of ±2000 basepairs around the transcription start site, we found 3 CArG motifs present upstream of human PDLIM7 (at positions +650, +654, +667) and one SRF binding site in the PLN gene, but no such motifs were found in either SYNPO2, LMOD1 or SYNM in this analysis. Another prediction program MotifMap, searching a wider region within 8000 basepairs around the transcription start, suggested regulation by several other transcription factors previously associated with SMCs or control of cell proliferation. Here, TEF1 and MAFA were predicted to regulate LMOD1; AP1 and SRF to regulate PDLIM7; TEF1 and SRF to regulate PLN; and CTCF and NEUROD to control SYNPO2 (full list in Supplementary Table VII).

Subcellular localization of SMC markers was also assessed in low-passage human SMCs (Figure 5C, additional images shown in Supplementary Figure VIII). CNN1, PDLIM7 and SYNPO2 were localized to the actin cytoskeleton by overlap with phalloidin staining; SYNM localized to cellular filopodia and to the cortical cytoskeleton; PLN exhibited nuclear staining while MYH11 and LMOD1 could not be detected in these cells. Taken together, our data confirm that SMCs maintain phenotypic plasticity in vitro and show that the expression changes and cytoskeletal localization of the selected genes strongly correlate with those of typical SMC markers in vitro, as initially observed in situ.

**Downregulation of LMOD1, SYNPO2, SYNM, PDLIM7 and PLN in response to inflammatory-, hemodynamic- and lipid stimuli**

Next, we explored processes relevant in the environment of an atherosclerotic lesion that may influence expression of the genes identified in our study. The expression of standard SMC markers as well as LMOD1, SYNPO2, SYNM, PDLIM7 and PLN was rapidly downregulated in human SMCs in vitro by stimulation with IFNg (Figure 6A). Downregulation of all genes was observed within 24h of IFNg treatment, whereas expression of PLN, PDLIM7, SYNM and SYNPO2 was suppressed already after 2h. These genes were also downregulated in human SMCs after 48-72hrs stimulation with oxLDL (in particular SYNPO2, LMOD1 and PLN, Figure 6B), which was validated by analyzing a public microarray dataset comparing cholesterol-loaded primary mouse aortic SMCs with baseline controls (GSE47744, Supplementary Figure IX)²⁰. In this model, the typical SMC markers ACTA2 and CNN1 were also downregulated, whereas the macrophage marker CD68 was upregulated. Finally, we analyzed expression of these genes in an in vitro model of SMC exposure to laminar shear stress, mimicking the exposure of the injured vessel surface to the hemodynamic forces of...
the flowing blood\textsuperscript{21}. In microarrays comparing shear stress vs. static conditions, we have previously observed apoptosis as an enriched pathway through activation of CASP3 (dataset accession nr GSE19909) and all genes (as well as other typical SMC markers) were also found to be downregulated in this model (Figure 6C).

Collectively, our results demonstrate that downregulation of \textit{LMOD1}, \textit{SYNPO2}, \textit{SYNM}, \textit{PDLIM7} and \textit{PLN} along with standard SMC markers, functionally relates to clinical symptoms of plaque instability, vascular injury, as well as to key molecular processes in atherosclerosis such as apoptosis, shear stress, inflammatory stimuli and lipid-uptake.

\textbf{Polymorphisms in} \textit{PDLIM7}, \textit{SYNPO2} and \textit{PLN} \textit{associate with surrogate markers of atherosclerosis}

In order to investigate the involvement of \textit{LMOD1}, \textit{SYNPO2}, \textit{PDLIM7}, \textit{SYNM} and \textit{PLN} in early processes shown to be predictive of carotid and coronary artery disease in humans, we examined the association of genetic variants in these loci with severity and rate of cIMT progression. Several variants in the \textit{PDLIM7}, \textit{SYNPO2} and \textit{PLN} genomic regions were found to be associated with cIMT phenotypes in a large cohort of high-risk subjects (n=3378, IMPROVE)\textsuperscript{22} after adjustment for age, gender and population stratification (Supplementary Table VIII). Variants rs11746443 and rs35716097 (\textit{PDLIM7}) associated with the maximum thickness of the common carotid artery (p=0.002) and the fastest cIMT progression (p=0.0009, p=0.0002, respectively), and variants rs67456868 (\textit{PLN}) and rs4833611 (\textit{SYNPO2}) were associated with the maximum common carotid artery thickness (p=0.00004, p=0.0007, respectively). Full functional information obtained from Haploreg for these variants is presented in Supplementary Tables IX and X. The \textit{SYNPO2} variant rs4833611 was located in the intronic region of the \textit{USP53} gene and by eQTL analyses marginally linked to \textit{SYNPO2} (p=0.09) and \textit{USP53} (p=0.02) gene expression in plaques. Of particular interest, \textit{PDLIM7} variant rs35716097 was predicted to constitute a putative binding site for the HNF4A transcription factor. The other \textit{PDLIM7} variant rs11746443 was localised in the genomic region of \textit{RGS14} and predicted to constitute the binding site for the HEY1 transcription factor, while its proxy (rs4075958, Rsquared=0.927, Dprime=0.963) was mapped within the putative binding site for the ETS1 transcription factor. By eQTL analysis in plaques, rs4075958 was found to be significantly associated with the expression of both \textit{PDLIM7} and \textit{RGS14} (p=0.007 and p=0.0002 respectively, Figures 7A and 7B) and the expression levels of both genes were strongly correlated (Pearson r=0.61, p<0.0001) (Figure 7C). \textit{PDLIM7} and \textit{RGS14} also appeared to be linked in a protein interaction network via actin cytoskeleton and markers of differentiated SMCs, SMTN and CNN2 (Figure 7D). Altogether, our results underline the possibility that genetic variants associated with \textit{PDLIM7} may be causal to altered intima-media phenotypes and predisposition to atherosclerosis.

\textbf{Silencing of} \textit{PDLIM7} \textit{leads to downregulation of other SMC markers and increased SMC proliferation}

Of the five genes that were identified, \textit{PDLIM7} emerged as one of the hub genes in the interaction network with other signature genes in atherosclerosis\textsuperscript{13}. Since \textit{PDLIM7} was causally implicated in atherogenesis at the genetic level, localised to SMC actin cytoskeleton and in addition, interconnected with other cytoskeletal proteins, we decided to mechanistically investigate its role in SMCs. Silencing \textit{PDLIM7} expression in human carotid SMCs in vitro, resulted in downregulation of other SMC markers (\textit{ACTA2}, \textit{MYH11}, \textit{LMOD1}, \textit{PLN} and particularly \textit{SYNPO2} by approximately 70% on the mRNA level). Cell adhesion and spreading on fibronectin were defective compared with controls, and proliferation was significantly increased in these cells as evaluated by BrdU incorporation (p<0.0001; Figure 8, Supplementary Figure X). These findings add mechanistic support to the notion that \textit{PDLIM7} is an important structural molecule in the regulation of SMC phenotype.
Discussion

A large biobank of carotid endarterectomies obtained from patients undergoing surgery for symptomatic or asymptomatic carotid stenosis was used to identify suppressed processes in atherosclerosis. We found molecular pathways related to SMC function and phenotype but also a panel of genes (SYNPO2, SYNM, LMOD1, PDLIM7, PLN) previously not associated with vascular disease, not only to be the most repressed in end-stage atherosclerosis but also in relation to clinical symptoms of plaque instability, both on the transcriptomic and proteomic level. We hypothesized that some of these genes may show early expression variations and demarcate the initiation of SMCs phenotypic switching. Most of these genes were linked to the SMC cytoskeleton, downregulated during neointima formation after rat carotid balloon injury, and polymorphisms in PDLIM7, PLN and SYNPO2 genomic regions were associated with cIMT phenotypes in high-risk subjects. In addition, expression of these genes was sensitive to predominant processes in the atherosclerotic lesion such as apoptosis, inflammation, hemodynamic stress, and lipid exposure. We propose that these SMC genes may improve definition of the phenotypic state of these cells in vascular disease and may be further explored in relation to the capacity of SMCs to contribute to plaque stabilization.

Previously, transition of SMCs from a contractile and quiescent phenotype into synthetic, matrix-producing and replicating cells has been widely accepted as a central feature in early atherogenesis and an essential part of lesion stability and repair. This process, where the typical contractile features of SMCs are lost, represents an example of disturbed vessel wall homeostasis in disease progression. However, it has become evident that these conclusions oversimplify the complexity of SMC function in vascular disease and that these phenotypes probably represent the extremes of a spectrum of intermediate phenotypes that may to various extents coexist in the vessel wall, as dictated by exposure to environmental cues affecting gene expression patterns. Recent studies have presented strong evidence that SMCs and macrophages can activate the same genes by demonstrating that 50% of foam cells within advanced human coronary artery lesions express the SMC marker SMA besides the macrophage marker CD68, while lineage tracing in mice confirmed that up to 80% of the lesion cells (including mesenchymal stem cells and macrophage-like cells) are SMC-derived. Here, we demonstrated that a number of SMC markers remain repressed on the protein level in stellate-shaped SMA+ cells of the fibrous cap, whereas expression was still detectable on the transcript level in situ, as previously reported by others. Together, these observations highlight the problem of correct SMC identification with respect to our understanding of human disease. Other studies seeking to establish the earliest determinants of SMC phenotypic switch have shown that e.g. mitochondrial fragmentation represents an early mark of SMC activation. Currently, changes in histone modifications, novel SMC-enriched transcription factors such as TCF21 and TET2, and epigenetic regulation of SMC phenotype by noncoding RNAs are also being intensively investigated. Nevertheless, our study highlights that we have not yet fully explored the transcriptomic landscape in relation to the plethora of SMC phenotypes and adds to elucidation of molecular signatures that characterize their plasticity.

Here, we confirmed that muscle-contraction, muscle-development and acto-myosin cytoskeleton were some of the most repressed categories in atherosclerotic tissue, including typical markers of SMCs as well as a number of genes previously poorly characterised in the context of SMC function. Synemin (SYNM) is an intermediate filament protein whose knockdown in saphenous vein SMCs in vitro leads to increased collagen production, downregulation of typical SMC markers and disassembly of actin fibers. Phospholamban (PLN) was previously immunolocalized to the nuclear envelope and sarcoplasmic reticulum of cardiac SMCs, where it has been proposed to regulate intracellular and intranuclear Ca2+ levels. It has also been shown that Ca2+ signaling pathways are altered in synthetic vascular SMCs, which possibly occurs in conjunction with PLN translocation from the nucleus to the other subcellular compartments. In a recent study, PLN mutations were linked
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to dilated cardiomyopathy, ventricular arrhythmias and interstitial fibrosis. Nanda V et al. described Leiomodin 1 (LMOD1) as a new SMC-restricted, myofilament-related, SRF/MYOCD target gene enriched in SMCs in embryonic and adult mouse tissues. Earlier, LMOD1 was predicted to belong to a ‘gene battery’ involved in SMC differentiation by a bioinformatics screen for regulators of conserved functional gene modules in mammals. Interestingly, Synaptopodin (SYNPO) and PDLIM proteins have been discovered as neuronal components and also investigated as adaptor molecules orchestrating actin-cytoskeletal organization in foot processes of podocytes, but sparsely linked to SMCs. Apart from these few publications, limited information exists about these genes in the literature up to date, and to the best of our knowledge, this study is the first to systematically examine their implication in human atherosclerosis and vascular remodelling.

SMCs are currently considered to be the main cell type responsible for intimal repair after balloon injury in the rat carotid artery, although cells of mesenchymal origin may also contribute. In this model, intimal hyperplasia develops in three major stages with initial SMC activation, replication and beginning of migration to the luminal surface during the first two days after injury. Between days two and five, SMCs colonise the intimal surface following activity related to chemoattractants and ECM degradation. In the next few weeks, the number of SMCs in the neointima continues to increase, but from one month after injury, SMC proliferation ceases, the cells become quiescent and regain ultrastructural features typical for the contractile state. Our results show that expression profiles for both typical SMC markers and for Synm, Pln, Lmod1, Synpo2 and Pdlim7 reflect these stages by gradual downregulation until five days after injury, followed by subsequent upregulation later as SMCs become quiescent and regain contractile features. In a similar fashion, immunohistochemical staining for typical SMC markers as well as for SYNM, PLN, LMOD1, SYNPO2, and PDLIM7 was detected in human intimal hyperplasia, especially in large PCNA-areas. Based on these results we hypothesize that SYNM, PLN, LMOD1, SYNPO2, and PDLIM7 functionally relate to the phenotypic state of SMCs.

Freshly isolated rat aortic SMCs seeded on fibronectin and cultured under serum-free conditions have previously been used to study the subcellular properties related to SMC phenotypic modulation in vitro. Under these conditions, interactions between fibronectin, integrin α5β1 and FAK-dependent intracellular signalling promote cell cycle entry and dedifferentiation into a synthetic state, accompanied by structural reorganisation and loss of myofilaments. Here, we observed that Synm, Pln, Lmod1, Synpo2 and Pdlim7 (as well as Acta2, Myocd, Myh11) were indeed downregulated in primary rat SMCs during the first days of culture on fibronectin, but reexpressed from about 5 days of culture, suggesting that SMCs retain their inherent plasticity in vitro. Several of the examined genes were localised to the actin cytoskeleton in human SMCs implying that they may be involved in reorganisation of cytoskeletal structures. Interestingly, while plasticity of SMCs and re-expression of target genes and proteins was apparent in human and rat intimal hyperplasia, expression of the proteins remained repressed in stellate-shaped SMA+ cells of the fibrous cap in carotid plaques. As discussed, this may either be due to a heterogeneous population of cells expressing SMA or repression of these genes in SMCs by disease specific factors such as inflammatory-, apoptotic-, or lipid mediators.

Therefore, in order to explore whether dominant processes in the atherosclerotic environment and in vascular disease in general may influence the genes of interest in our study, we investigated the expression of SYNM, PLN, PDLIM7, LMOD1 and SYNPO2 as well as other typical SMC markers in SMCs exposed to disease-associated stimuli in vitro. To summarize, while we have not yet fully dissected which specific stimulus or combination of stimuli may repress expression of SYNM, PLN, PDLIM7, LMOD1 and SYNPO2 in atherosclerosis and vascular remodelling, we showed that they were downregulated by inflammatory stimuli and cholesterol-uptake, and in response to shear stress (and apoptosis). In support of these observations, exposure to lipids has previously been associated with
dramatic effects on SMC phenotype and transdifferentiation into CD68+ macrophage-like foam cells, as also demonstrated in our study.\textsuperscript{20}

Intima–media thickness of extracranial carotid arteries, measured by ultrasound is a commonly accepted non-invasive marker of subclinical atherosclerosis. Several studies have established that cIMT changes over time are associated with vascular risk factors\textsuperscript{22} and prediction of vascular events both in subjects with plaques at baseline and in those without. Here, genetic variants in \textit{PDLIM7}, \textit{SYNPO2} and \textit{PLN} showed association with cIMT measurements, suggesting that these genes could have a causal role in carotid disease. Of note, \textit{SYNPO2} variants were located in the intron of the \textit{USP53} gene and marginally linked to expression in BiKE atherosclerotic plaques. \textit{USP53} (Ubiquitin Specific Peptidase 53) is a poorly studied protein, highly expressed in the heart muscle and found to be genetically associated with the Cantu syndrome, a rare condition characterized clinically by hypertrichosis, cardiomegaly and bone abnormalities\textsuperscript{49}. Of particular interest, \textit{PDLIM7} SNPs linked to fastest-IMTmax-progression were shown to influence expression of \textit{PDLIM7} in plaques and predicted to constitute binding sites for transcription factors previously implicated in cardiovascular development, SMC migration, and SMC proliferation in response to cytokine stimulation\textsuperscript{50, 51}. One of these SNPs was positioned in the intronic region of the \textit{RGS14} gene, and interestingly, the expression of \textit{RGS14} also strongly correlated with the expression of \textit{PDLIM7} in plaques. \textit{RGS14} has been shown to act as a positive modulator of microtubule polymerisation and spindle organization during cell division by integrating G protein and MAPK signaling pathways\textsuperscript{52, 53}. It inhibits PDGF-stimulated ERK1/ERK2 phosphorylation and may indirectly interact with \textit{PDLIM7} via the actin-cytoskeleton.

The importance of \textit{PDLIM7} for SMC phenotype was confirmed by silencing experiments that resulted in perturbed cytoskeletal structure, adhesion and spreading as well as SMC proliferation. Previous studies in other cell types have shown similar effects of \textit{PDLIM7} knock-down on proliferation (i.e. periodontal ligaments\textsuperscript{54}) and studies of other PDLIM family members have shown that they can directly interact with actin-cytoskeleton proteins such as α-actinin-4 to stabilise actin fibres\textsuperscript{39}. Similarly, missense mutations of \textit{ACTA2} in humans are associated with diminished gene expression, defective actin-filaments and actin-based spreading in SMCs, and formation of occlusive lesions due to increased SMC proliferation and intimal hyperplasia\textsuperscript{55, 56}. Overall, our findings suggest an important structural and mechanistic role for \textit{PDLIM7} in SMCs, with possible genetic influence on disease development.

Because the BiKE cohort comprises only late-stage lesions and cannot provide information about gene expression variations during atheroprospergession, expression data was complemented with immunohistochemistry on aortic lesions collected from different stages of atherosclerotic disease. Of note, PCNA that was used as a proliferation marker in the immunohistochemical analysis, has been reported to overestimate the number of replicating cells. Consensus is lacking regarding the selection of appropriate control tissues, and in the BiKE study, control vessels contained outer media that is not included in the endarterectomy samples. Furthermore, the discovery approach in our study was based on microarrays and the complexity of microarray data was reduced by pathway analyses and construction of functional networks where genes were clustered based on biological functions or protein interactions. While this method is limited to semantic mining of existing knowledge from published literature and databases, it still permits for discovery of poorly explored genes in a certain context.

In conclusion, using a systems biology approach by integrating transcriptomic, in situ, in vivo, in vitro and genetic studies we were able to overcome these limitations and discover several novel candidates that demarcate early phenotypic modulation of SMCs in vascular disease. In perspective, the full knowledge of key expression signatures is likely to help us derive a better definition of various SMC phenotypes that coexist in the vessel wall, and provide potential targets for prevention and therapy in vascular disease.
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Disclosures

Authors have no competing interests to declare.
References


**Significance**

A large biobank of carotid endarterectomies obtained from patients undergoing surgery for symptomatic or asymptomatic carotid stenosis was utilized to uncover genes and mechanisms repressed in atherosclerosis. Results demonstrated enrichment of molecular pathways related to smooth muscle cell (SMC) function and identified a panel of downregulated SMC genes previously not associated with vascular disease. These genes (*SYNPO2, SYNM, LMOD1, PDLIM7 and PLN*) were related to the SMC cytoskeleton, they were transiently downregulated during neointima formation after rat carotid balloon injury, and polymorphisms in *PDLIM7, PLN* and *SYNPO2* were associated with surrogate markers of atherosclerosis in high-risk subjects without symptoms of cardiovascular disease. Our work emphasizes the significance of SMC phenotypic modulation in atherosclerosis. In addition, these newly described SMC genes may improve definition of the phenotypic state of these cells in vascular disease and may be related to the capacity of SMCs to contribute to stabilizing processes in atherosclerotic lesions.

**Highlights**

- An integrative approach utilizing large biobanks of carotid endarterectomies from patients undergoing surgery for symptomatic or asymptomatic stenosis was utilized to uncover mechanisms repressed in atherosclerosis.
- A combination of transcriptomic and proteomic profiling revealed that SMCs related molecular categories were the most downregulated in plaques and identified five genes previously not associated with atherosclerosis.
- *SYNPO2, SYNM, LMOD1, PDLIM7 and PLN* were related to SMC cytoskeleton, transiently downregulated during neointima formation after rat carotid balloon injury, by PDGFB and IFNg, exposure to shear flow stress and oxLDL loading.
- Polymorphisms in *PDLIM7* were associated with surrogate markers of atherosclerosis in high-risk subjects, while it's silencing in vitro led to downregulation of SMC markers, disruption of the actin cytoskeleton, decreased cell spreading and increased proliferation.
- The newly described SMC genes may improve definition of the phenotypic state of these cells in vascular disease and relate to their capacity to contribute to stabilizing processes in atherosclerotic lesions.
Figure Legends

Figure 1. LMOD1, SYNPO2, SYNM, PDLIM7 and PLN were downregulated in carotid plaques. Microarray profiles comparing n=127 carotid plaques vs. n=10 normal arteries were used in discovery phase for analysis of downregulated genes and pathways. Arrowheads showing SMA+ smooth muscle cells (SMCs) in the media of normal artery, remaining media at the plaque periphery and fibrous cap. Images were taken with 4x objective. (A). Co-expression and co-interaction network of genes clustered in pathways 'muscle contraction', 'muscle development', 'actin cytoskeleton' and 'myofibril cytoskeleton' shows the presence of typical markers of SMCs in these categories (i.e. CNN1, SMTN, TAGLN, ACTA2, MYH11) as well as other potentially interesting genes of which some were selected for further investigations. Network weighted for closeness in biological function based on publically available data (B). PDLIM7, LMOD1, SYNPO 2, SYNM and PLN were among the most downregulated genes in microarrays from plaques (CP) vs. normal arteries (NA) and symptomatic (S) vs. asymptomatic (AS) patients (C) and strongly correlated to standard markers of SMCs (D). Plots showing log mean±standard deviation in C and Pearson correlations in D.

Figure 2. Selected candidates were localised to differentiated smooth muscle cells and reduced in late-stage plaques. By immunohistochemistry, Myh11 (red) was present only in normal carotid arteries while Calponin (red) and SMA (green) were detectable in normal arteries, in plaques as well as in human intimal hyperplasia tissue with large PCNA- areas (arrowheads, A). The identified SMCs markers (red) were all localised to SMCs in the normal carotid artery (left column panels, insets show higher magnification). Pdlim7 and Synm were also present in subintimal SMA+ cells at the plaque periphery and Pdlim7 was the only one still detectable in SMA+ cells in the fibrous cap. Signal for Lmod1, Synpo2 and Pln was lost in plaques. Abundant staining for Pdlim7 and Synm was seen in restenosis tissues, and Synpo2, Lmod1 and Pln were also observed in PCNA- areas (B). Images were taken with 10x objective, insets show 40x magnification.

Figure 3. Expression of LMOD1, SYNPO2, SYNM, PLN and PDLIM7 strongly correlated to typical markers of SMCs during the course of rat carotid artery injury and healing response. By microarray profiling, the identified SMC proteins and typical markers of SMCs were downregulated in early phases up to day 5 after vessel injury and gradually upregulated in later phases in intimal hyperplasia after rat carotid balloon injury (A, B). Expression correlations of LMOD1, SYNPO2, SYNM, PLN and PDLIM7 with typical SMCs markers were significant and strongly positive in this model (mostly Pearson r>0.8), while they were negative with PDGFB, IGF1 and TGFβ1 (C).

Figure 4. Lmod1, Synpo2, Synm, Pln and Pdlim7 were localised to SMCs in intact rat carotid artery and reduced in response to injury. By IHC the loss of Calponin (red) from highly proliferative PCNA+ SMCs layers in the injured rat artery closer to the lumen was observed (arrowheads), while staining was still present in deeper medial layers at day 5. At 12 weeks after injury Calponin was again abundant in the mature intima with less proliferative cells in the deeper layers but absent from luminal PCNA+ layers (arrowheads, A). The signal for identified SMCs markers (red) was completely absent at day 5 with gradual reappearance from the medial SMCs in tissues with pronounced intimal hyperplasia at 12 weeks after injury (B). Images were taken with 20x objective, insets show higher magnification (100x) of the media.

Figure 5. LMOD1, SYNPO2, SYNM, PLN and PDLIM7 were expressed by differentiated SMCs in vitro and localised to actin cytoskeleton. By flow cytometry 90% of the primary rat aortic cells were SMA+ on day 0 (after overnight collagenase treatment, top panel, A) as well as 7 days upon isolation when cultured in serum-free medium (second panel from top, A). A subpopulation of cells with lower SMA+ signal was identified in cultures at day 0, as well as 7 days upon isolation when stimulated with PDGFB (arrows, top and third panel, A). Bottom panel in A shows overlap of the upper 3 panels indicating the change in the SMA signal during 7 days of culture. By qPCR analysis rSMCs showed downregulation of
Markers of smooth muscle cells

conventional and identified SMCs markers at day 3 upon isolation and a trend towards upregulation after 5 days in culture. Graphs showing mean fold change ± SEM, ANOVA p-values, results representative of 3 independent primary cell isolations (B). In low-passage primary human carotid SMCs Pdlim7, Synm and Synpo2 colocalised with the actin cytoskeleton (as shown by phalloidin staining in red), Pln showed nuclear localisation and the signal for Lmod1 was beyond detection. For comparison, Calponin was localised to actin cytoskeleton (C). Images were taken with 100x objective.

**Figure 6. SMCs markers were downregulated in relation to inflammation, lipid-loading and hemodynamic stress.** CNN1, PDLIM7, LMOD1, SYNPO2, PLN and SYNM were rapidly downregulated by IFNg treatment of cultured human carotid SMCs (A) and stimulation with oxLDL similarly resulted in downregulation of these genes (B). These SMC genes were also repressed in primary rat aortic SMCs by exposure to laminar shear stress (dataset GEO accession nr GSE19909, C). Plots show mean fold change ± SEM, p-values from T-test or ANOVA when appropriate. Results are representative of 3 independent experiments.

**Figure 7. Polymorphism in the PDLIM7 genomic region associated with carotid intima-media thickness affects its expression in plaque tissue.** By eQTL analysis variant rs4075958 was associated with the mRNA expression of both PDLIM7 and RGS14 in plaque tissue (A, B) and the expression levels of these two genes were strongly correlated (C). Functional network coupling based on protein-protein interactions links Pdlim7 and Rgs14 via actin cytoskeleton proteins (D). Plots in A and B show median with minimum and maximum.

**Figure 8. Silencing of PDLIM7 leads to downregulation of other SMC markers and increased SMC proliferation.** PDLIM7 expression was silenced in human SMCs in vitro using siRNA (Crystal Violet staining, A), which resulted in downregulation of several other SMC markers (B), increased cell proliferation (as evaluated by BrdU incorporation) and impaired cell spreading/adhesion ability (C). Plots show mean ± SEM.
Figure 1.
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