Urokinase Receptor Associates With Myocardin to Control Vascular Smooth Muscle Cells Phenotype in Vascular Disease

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Objective—The urokinase-type plasminogen activator (uPA) and its specific receptor (uPAR) are a potent multifunctional system involved in vascular remodeling. The goal of the study was to unravel the mechanisms of uPA/uPAR-directed vascular smooth muscle cell (VSMC) differentiation.

Methods and Results—Using cultured human primary VSMCs, we identified a new molecular mechanism controlling phenotypic modulation in vitro and in vivo. We found that the urokinase-type plasminogen activator receptor (uPAR) acts together with the transcriptional coactivator myocardin to regulate the VSMC phenotype. uPAR, a glycosylphosphatidylinositol-anchored cell-surface receptor family member, undergoes ligand-induced internalization and nuclear transport in VSMCs. Platelet-derived growth factor receptor β and SUMOylated RanGAP1 mediate this trafficking. Nuclear uPAR associates with myocardin, which is then recruited from the promoters of serum response factor target genes and undergoes proteasomal degradation. This chain of events initiates the synthetic VSMC phenotype. Using mouse carotid artery ligation model, we show that this mechanism contributes to adverse vascular remodeling after injury in vivo. We then cultured cells on a microstructured biomaterial and found that substrate topography induced uPAR-mediated VSMC differentiation.

Conclusion—These findings reveal the transcriptional activity of uPAR, controlling the differentiation of VSMCs in a vascular disease model. They also suggest a new role for uPAR as a therapeutic target and as a marker for VSMC phenotyping on prosthetic biomaterials. (Arterioscler Thromb Vasc Biol. 2012;32:110-122.)

Key Words: plasminogen activators ■ receptors ■ vascular muscle ■ remodeling ■ urokinase

A disrupted balance between vascular smooth muscle cell (VSMC) differentiation and proliferation occurs in atherosclerosis, postangioplasty restenosis, arterial hypertension, and asthma. The synthetic VSMC phenotype is characterized by loss of contractility and abnormal proliferation, migration, and matrix production. By reinducing differentiation marker genes, VSMCs reacquire their contractile phenotype. Deregulation of this plasticity contributes to the pathogenesis of vascular disease by a process known as negative vascular remodeling. An advantage can be gained by switching VSMCs from the synthetic to a contractile phenotype, particularly in those VSMCs residing on biosynthetic materials. The mechanisms that control VSMC plasticity and the switch between synthetic and contractile phenotypes are incompletely understood.

The urokinase-type plasminogen activator (uPA) and its specific receptor (uPAR) are a remarkably potent multifunctional system involved in vascular remodeling. Both uPA and uPAR are induced after vascular injury and promote neointima formation in atherosclerosis. The uPA/uPAR system controls VSMC migration and proliferation and fibrin deposition, and it interferes with wound healing. The uPAR also influences remodeling-related inflammation and uPAR-dependent intracellular signaling regulate the epithelial-mesenchymal transition and fibroblast-to-myofibroblast differentiation. We observed earlier that uPAR is an active regulator of VSMC phenotypic changes. We suggested that upregulated uPAR expression at vascular injury sites could serve for VSMC transition from their physiological contractile to the pathophysiological synthetic phenotype. However, the mechanism by which uPAR may interfere with regulation of contractile protein expression is not known.

uPA is a proteolytic enzyme mediating plasminogen activation. Because uPAR is linked to the outer membrane leaflet by a glycosylphosphatidylinositol anchor and has no
transmembrane and cytoplasmic domains, uPAR’s diverse biological functions are strictly dependent on its interactions with other proteins. Thus, the uPAR “interactome” includes membrane proteins, soluble factors present in the pericellular space, and partners in the extracellular matrix. As a result, uPAR activates numerous intracellular signaling molecules leading to proliferative, migratory, adhesive, and apoptotic responses. In VSMCs, uPAR is associated with platelet-
derived growth factor receptor β (PDGFRβ), which serves as a transmembrane adaptor to mediate intracellular signaling and cellular responses. uPAR also controls VSMC proliferative phenotype through the transcription factor Stat1, whereas uPAR signals a migratory behavior through Tyk2, phosphatidylinositol 3-kinase, and the Rho family of proteins.

VSMC differentiation marker genes are regulated by serum response factor (SRF), a transcription factor that binds to conserved promoter CArG boxes. Myocardin is a recently identified coactivator of SRF-dependent transcription exclusively expressed in cardiac and VSMC lineages that is required for VSMC differentiation. Myocardin selectively induces expression of CArG-dependent VSMC marker genes, including smooth muscle α-actin (SMA), smooth muscle myosin heavy chain, SM22α, and calponin. Mice lacking myocardin die during midembryogenesis from a lack of differentiated VSMCs. Remarkably, forced expression of myocardin in fibroblasts is sufficient to activate the VSMC differentiation program. We explored the hypothesis that myocardin is involved in the uPAR-directed VSMC phenotypic changes. We found that uPAR inhibits myocardin-dependent smooth muscle gene expression and transcriptional activity. Unexpectedly, this mechanism requires uPAR nuclear translocation and its direct interaction with myocardin in vitro and in vivo. We identify the uPAR-myocardin axis as...
Figure 3 Urokinase-type plasminogen activator receptor (uPAR) and myocardin associate in cell nucleus in a urokinase-type plasminogen activator (uPA)-dependent manner. A, Vascular smooth muscle cells (VSMCs) stimulated with uPA for 1 and 2 hours.
a new regulatory mechanism of the VSMC differentiation and dedifferentiation program on vascular remodeling.

**Methods**

**Cell Culture and Transfections**

Human VSMCs were isolated from umbilical arteries using the explant technique in VascuLife SMC cell culture medium (CellSystems Biotechnologie Vertrieb GmbH). VSMCs were used between passages 2 and 4. Aortic VSMCs from uPA+/−, uPAR+/− and corresponding wild-type control animals were isolated by enzymatic digestion as described earlier.13 Primary VSMCs were transfected using Amaxa Nucleofector I (Lonza Group Ltd) and the Human Primary Smooth Muscle Cells Basic Nucleofection Kit (Lonza Group Ltd) according to the manufacturer's instructions. Lentiviral infection of mouse VSMCs was performed as described.24 The constructs to express luciferase under SMA and SM22α promoters were a generous gift of Dr G. Owens (University of Virginia School of Medicine). Constructs to express glutathione S-transferase–fusion mutants of myocardin were kindly given by Dr Jiiliang Zhou (Albany Medical College). Vector for expression of DDK-tagged myocardin was purchased from Origene (RC229055).

**Biochemical Methods**

Subcellular fractionation was performed using Subcellular Protein Fractionation Kit (Pierce, Thermo Scientific). Coimmunoprecipitation (co-IP) of nuclear proteins was studied using the Nuclear Complex Co-IP kit (Active Motif). Biotinylation of surface proteins was performed using 2 mg/mL sulfo-N-hydroxysulfo-nimicid SS-biotin in PBS. Biotinylated cells were then stimulated with uPA and incubated at 37°C to allow internalization. Reduction of proteins remained on the plasma membrane allowed detection of internalized proteins only. Luciferase activity was measured using Promega ONE-Glo luciferase assay system according to the manufacturer’s instructions. 20S proteasome activity was measured fluorometrically using the assay kit from Cayman Chemical Company using SUC-LLVY-AMC as a substrate.

**Chromatin Immunoprecipitation Assay and Reverse Transcription–Polymerase Chain Reaction**

Chromatin immunoprecipitation analysis was performed using SimpleChIP Enzymatic Chromatin IP kit (Cell Signaling Technology). Anti-SRF antibody was used for immunoprecipitation, and then SRF association with SMA and smooth muscle myosin heavy chain promoters was estimated using SYBR Green reverse transcription–polymerase chain reaction. The total RNA for quantitative reverse transcription–polymerase chain reaction was isolated from VSMCs using the RNeasy miniprep kit (Qiagen). Real-time quantitative reverse transcription–polymerase chain reaction was performed on a LightCycler 480 Real-Time PCR System using LightCycler 480 RNA Master Hydrolysis probes (Roche Diagnostics GmbH).

**Immunofluorescence**

For immunocytochemical staining, we relied on paraformaldehyde-fixed, Triton X-100–permeabilized cells cultured on glass coverslips. As DNA stains, we used either DraQ5 or 4',6-diamidino-2-phenylindole. The confocal cell images were captured using a Leica LCS-SP2 AOBS confocal microscope (Leica Microsystems). For the details of cell staining for fluorescence resonance energy transfer (FRET) analysis and images acquisition and analysis, please refer to the supplemental material, available online at http://atvb.ahajournals.org.

**Immunocytochemistry and Immunohistochemistry**

Cells were blocked with 3% BSA and labeled with primary antibody (2 hours at room temperature) and fluorescently labeled secondary antibodies (1 hour at room temperature). As DNA stains, either DraQ5 or 4',6-diamidino-2-phenylindole was used. Frozen sections were blocked with 5% goat serum and incubated with anti-uPAR (M-17) antibody (Santa Cruz Biotechnology) in blocking solution overnight and chicken anti-goat Alexa 488 antibody for 1 hour at room temperature. Cells nuclei were labeled with Draq5, and slides were mounted using Aqua Poly/ Mount medium (Polysciences).

**Mouse Carotid Artery Ligation**

The mouse carotid artery ligation model was performed as described.25 Animal experiments were approved by the regulatory board for animal welfare of the state of Niedersachsen, Germany.

**Microstructured Substrates**

Microstructured cell substrates were fabricated from organically modified ceramics (ORMOCER, Microresist GmbH), a silica-based inorganic-organic hybrid polymer, by 2-photon polymerization with femtosecond pulses.26,27 VSMCs cultivated on plane and structured ORMOCER were fixed and stained similarly to cells cultivated on glass coverslips.

**Statistics**

Data were analyzed for statistical significance using the 2-tailed Student t test for independent samples (OriginPro 8 SRO). Data are presented as mean±SEM. Differences were considered statistically significant at a value of P<0.05.

For a detailed description, please refer to the supplemental material.

**Results**

**Myocardin Is Involved in uPAR-Directed VSMC Phenotypic Changes**

Our previous observations showed that uPAR is involved in VSMC phenotypic regulation. Downregulation of uPAR expression using small interfering RNA gene silencing (uPARsi) in human VSMCs (Supplemental Figure 1A) resulted in increased activity of smooth muscle promoters10 and increased expression of contractile proteins mRNAs, such as SMA and calponin (Figure 1A, left). In vivo, infiltrating cells serve as a source of uPA at sites of vascular injury. In cell culture, VSMCs produce both uPA and uPAR.9 We reasoned that the effect of receptor silencing could be explained by an inhibition of autocrine uPA/uPAR action directed to cell switch to synthetic phenotype and proliferation. We then used

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**Figure 3 (Continued).** were then fixed and stained for myocardin (Alexa 488) and uPAR (Alexa 594). The plots in the bottom row show intensity profiles of myocardin and uPAR images along the lines indicated by arrows. Images of uPAR and myocardin used for the analysis are shown in Supplemental Figure II A, B, uPA induced intracellular redistribution of uPAR and myocardin. Subcellular fractions were obtained using a subcellular fractionation kit. The distribution of protein markers of different subcellular fractions obtained during cell fractionation is shown in Supplemental Figure II B. C, Coinmunoprecipitation (co-IP) of myocardin and uPAR from the whole cell lysates of cells stimulated with uPA for 1 hour. D, uPAR coimmunoprecipitated with myocardin from nuclear extract using nuclear complex co-IP kit; see also Supplemental Figure II J. E, Fluorescence resonance energy transfer (FRET) was observed between myocardin and nuclear uPAR. The left and the middle panels show images of cells stimulated with uPA for 1 hour and stained for uPAR and myocardin, respectively. The right panel shows the normalized FRET signal (F_R) distributions over cells indicated by color coding. F, Lysates of VSMCs stimulated with uPA for 1 hour were used for pull-down assay with glutathione S-transferase (GST)–myocardin mutants. Domains structure of the mutants is shown at the bottom. NTD indicates N-terminal domain; +++, basic domain; Q, poly(Q) domain; LZ, leucine zipper domain; TAD, transcriptional activation domain; mut, mutant. G, Nuclear co-IP was performed from DDK-myocardin overexpressing cells. Scale bars=10 μm.
Figure 4. Urokinase-type plasminogen activator (uPA)–induced internalization of uPA receptor (uPAR) is required for myocardin degradation. A, Vascular smooth muscle cells (VSMCs) were subjected to biotinylation of cell surface proteins and then stimulated with uPA for 1 hour. In reduced control cells, cell-surface bound biotin was reduced before uPA stimulation. Biotinylated uPAR was immunoprecipitated from nuclear extracts using anti-uPAR antibody and visualized using streptavidin-conjugated horseradish peroxidase (SV-HRP) (upper panel) and anti-uPAR antibody after membrane stripping (lower panel). IP indicates immunoprecipitation. B, Myocardin was
small interfering RNA gene silencing to downregulate uPA expression (uPAsi) (Supplemental Figure 1B) and observed effects similar to uPARsi (Figure 1A, left). Accordingly, addition of exogenous uPA decreased the expression of contractile proteins (Figure 1A, right).

Because uPAR exhibits also ligand-independent functions, we downregulated uPAR expression in aortic VSMCs from uPA−/− mice with lentiviral-based uPARsi to confirm that changes in VSMC phenotype are directed by uPA-dependent uPAR activation. The expression of contractile proteins was elevated in VSMCs isolated from uPA−/− mice compared with wild-type control cells (Figure 1B). Although expression of contractile proteins was slightly increased after uPARsi in uPA−/− cells, receptor silencing had no additive effect. This result suggests that uPA and uPAR act together to downregulate contractile protein expression in VSMCs.

Myocardin is expressed in human umbilical artery VSMCs, and its downregulation indeed decreased the expression of contractile proteins, such as SMA and calponin (Supplemental Figure 1C–F). To investigate the potential role of myocardin in uPA/uPAR-directed VSMC phenotypic changes, we first examined effects of uPAR downregulation on myocardin. Western blotting and reverse transcription–polymerase chain reaction analysis showed that the protein level of myocardin was markedly upregulated in uPARsi cells, whereas uPA stimulation decreased it (Figure 1C and 1D).

mRNA level of myocardin was not upregulated by uPARsi but was decreased after uPA treatment in a uPAR-dependent manner (Supplemental Figure IG). Similarly, myocardin expression in VSMCs isolated from uPA−/− mice was comparable to wild-type and downregulated by uPA in a uPAR-dependent manner (Supplemental Figure IG).

To determine whether or not the uPA/uPAR system could directly affect myocardin-dependent transcriptional activity, luciferase assays were performed with SMA and SMA22α promoters in cells stimulated with uPA. uPA decreased promoter activity in a uPAR-dependent manner (Figure 1E).

Myocardin transactivates smooth muscle marker gene expression by physically interacting with SRF on promoters of smooth muscle genes. Therefore, decrease of myocardin protein level should result in changes of SRF transcriptional activity. We addressed this question by studying myocardin/ SRF interaction using co-IP analysis. We observed a time-dependent dissociation of myocardin/SRF complex in response to cell stimulation with uPA (Figure 1F). To confirm further the involvement of myocardin in uPA/uPAR-mediated events, we overexpressed human DDK-tagged myocardin in VSMCs (Supplemental Figure 1H and I). In myocardin-overexpressing VSMCs, uPA failed to downregulate promoter activity of contractile proteins (Figure 1G).

Accordingly, the decreased SRF association with smooth muscle gene promoters after uPA treatment observed in chromatin immunoprecipitation assay was also prevented by myocardin overexpression (Figure 1H).

Together, the above data demonstrate that uPA and uPAR affect the formation of the SRF/myocardin/CArG complex.

**uPAR Promotes Myocardin Proteasomal Degradation**

To address the mechanisms used by the uPA/uPAR system to regulate the myocardin protein levels, we performed immunocytochemical staining of myocardin in human VSMCs (Figure 2A). When the cells were stimulated with uPA, myocardin redistributed within nucleus and accumulated in nuclear bodies that look like clastosomes that are enriched in proteasomal subunits. When stained with 26S proteasome antibodies, these structures indeed revealed colocalization of myocardin and proteasomes (Figure 2B). The number of cells containing clastosome-like structures increased with uPA stimulation (Figure 2C). When the cells were treated with the proteasome inhibitor AM-114, the uPA-induced decrease of myocardin protein was abolished (Figure 2D). The data suggest that 30 to 60 minutes after uPA application the SRF/myocardin complex dissociates and myocardin is degraded. Accordingly, AM-114 treatment prevented downregulation of contractile protein expression in TaqMan analysis (Figure 2E). Because proteasomal degradation of myocardin has been reported, we studied whether or not uPA induces myocardin ubiquitination and found that it was increased by uPA (Figure 2F). These data support our assumption that the effect of uPAR on myocardin protein might be mediated through proteasomal degradation.

**uPAR Translocates to the Cell Nucleus and Associates With Myocardin**

We performed immunocytochemistry and unexpectedly observed that uPA induced uPAR translocation to cell nucleus (Figure 3A and Supplemental Figure II). Similar results were obtained using mouse aortic VSMCs (Supplemental Figure IIIA). To characterize intracellular redistribution of uPAR and myocardin in response to uPA further, we stained human VSMCs and then created image intensity profiles using ImageJ software. uPA treatment led first to uPAR translocation to cell nucleus and then to the clastosomes, where uPAR colocalized with myocardin (Figure 3A). We used confocal microscopy to localize uPAR at serial planes of the cell nucleus (Supplemental Figure IIC). 3D reconstruction of clastosome-containing cell nuclei stained for uPAR and myocardin showed colocalization of proteins in clastosome-like structures, with uPAR being localized in the core of the

**Figure 4 (Continued).** immunoprecipitated from nuclear extract of VSMCs after biotinylation and stimulation with uPA for 1 hour. uPAR in immunoprecipitates was detected using SV-HRP. C, uPA-induced degradation of myocardin in VSMCs treated with 5 μmol/L of amiloride or 10 μmol/L of chlorpromazine (45 minutes) before 3 hours of uPA stimulation. The lower panel shows quantification of the Western blotting data. D, Myocardin ubiquitination in VSMCs treated with amiloride or chlorpromazine before uPA stimulation assessed by immunoprecipitation. E, uPA- or amino-terminal fragment of uPA (ATF)-stimulated VSMCs (20 minutes) stained for early endosomal antigen (EEA-1) (Alexa 488) and uPAR (Alexa 594). Arrows in the middle panel show colocalization of EEA-1 and uPAR. F, uPA-stimulated VSMCs (20 minutes) stained for platelet-derived growth factor receptor β (PDGFRβ) (Alexa 488) and uPAR (Alexa 594). Arrows in the middle panel show colocalization of PDGFRβ and uPAR in early endosomal compartment. G, Myocardin degradation after 3 hours of uPA stimulation in VSMCs treated with 10 μmol/L AG 1295 (left panels) and VSMCs nucleofected with PDGFRβ siRNA (right panels). The lower panels show quantification of the Western blotting data. Scale bar=10 μm.
structure. Video obtained from serial confocal images is available as the supplemental video. These data clearly demonstrate ligand-induced uPAR nuclear translocation in VSMCs. Interestingly, this response to uPA stimulation was observed in cells at passages 2 to 3 that were stained positively for SMA-containing fibers. The number of such cells decreased progressively during cell culturing.

Next, we addressed uPA-induced uPAR and myocardin redistribution by performing isolation of subcellular fractions using a subcellular fractionation kit. We isolated cytosolic, membrane, soluble nuclear, and chromatin-bound nuclear extracts (Supplemental Figure IIIB). Cells stimulation with uPA induced redistribution of uPAR from membrane fraction to soluble nuclear extract, which was accompanied by redistribution of myocardin from chromatin-bound nuclear extract to soluble nuclear extract (Figure 3B).

We observed co-IP of uPAR and myocardin from whole cell lysates (Figure 3C). We then performed co-IP on cytosolic fractions and nuclear extracts. The enzymatic nuclear extraction process (using a nuclear complex co-IP kit) maintains chromatin-bound and free protein complexes contained in the nucleus (Supplemental Figure IJ). These experiments revealed uPA-induced myocardin-uPAR association in the nuclear fraction but not in the cytoplasmic fraction (Figure 3D).

To directly address the possibility of uPAR association with myocardin, uPAR-myocardin interaction, we used FRET. Nuclear translocation of uPAR was accompanied by the appearance of a FRET signal between myocardin and uPAR in cell nucleus and particularly in clastosomes (Figure 3E), confirming the direct association of the 2 proteins in cell nucleus. To characterize the interaction between uPAR and myocardin, we performed a glutathione S-transferase pull-down assay using glutathione S-transferase–myocardin fusion protein mutants. We observed that only the N-terminal part of myocardin, particularly the N-terminal domain, was required for interaction with uPAR from cell lysates of uPA-stimulated VSMCs (Figure 3F). Accordingly, in nuclear co-IP from VSMCs overexpressing DDK-tagged myocardin, we observed its very strong association with uPAR after uPA stimulation in nuclear co-IP using anti-DDK antibody (Figure 3G).

Cell Surface uPAR Undergoes Endocytosis and Nuclear Translocation

The above data demonstrated that binding of extracellular ligand uPA induced uPAR endocytosis and accumulation in nucleus. At least 2 internalization pathways have been described for uPAR. The ternary uPAR/uPA/plasminogen activator inhibitor-1 complex associates rapidly to low-density lipoprotein receptor–related protein-1 and is efficiently removed from the cell surface via clathrin-coated pits. Recent studies showed that uPAR might also be endocytosed by a macropinocytic mechanism coupled with rapid recycling to the cell surface independently of ligands. To clarify which mechanisms are responsible for uPAR intracellular translocation in VSMCs, we first looked for evidence that membrane pool of uPAR translocates to the nucleus. We labeled cell surface proteins using sulfo-N-hydroxysulfosuccinimide-SS biotin. Reduction of the proteins after cell stimulation with uPA allowed us to distinguish internalized protein exclusively. Internalized uPAR was immunoprecipitated from nuclear extract using the nuclear complex co-IP kit followed by Western blotting with streptavidin-conjugated horseradish peroxidase (Figure 4A). In cells subjected to biotin reduction before uPA stimulation, nuclear uPAR was not detected by streptavidin-conjugated horseradish peroxidase, but it was detected with uPAR antibodies (Figure 4A). Biotinylated uPAR was also co-IP with myocardin in nuclear co-IP (Figure 4B). Samples subjected to reduction of cell-surface protein-bound biotin immediately after labeling contained no myocardin-associated biotinylated proteins. These results indicate that cell surface uPAR translocates to the cell nucleus in the presence of uPA.
Figure 6. Urokinase-type plasminogen activator receptor (uPAR) mediates injury-induced dedifferentiation of vascular smooth muscle cells (VSMCs) in vivo. A, Hematoxylin/eosin staining of sham and ligated carotid artery of wild-type (WT) and uPAR−/− mice 3 (upper panels) and 28 (lower panels) days after vessel ligation (magnification ×40). B, Quantification of media thickening of ligated carotid artery of WT and uPAR−/− mice 3 days after vessel ligation. C, Quantification of the intima/media ratio of control and ligated carotid artery of WT and uPAR−/− mice 28 days after ligation. D, Expression of uPAR mRNA in control and ligated carotid artery of WT mice analyzed by TaqMan. 18S RNA was used for normalization. uPAR−/− arteries showed no uPAR expression. E, Cryosections of control and ligated carotid artery of WT mice 24 hours after ligation were stained for uPAR (Alexa 488). Draq5 was used as a nuclear marker.
To address the nature of uPAR endocytosis, we used chlorpromazine, which inhibits the clathrin-dependent endocytic pathway, and amiloride, an inhibitor of macropinocytic pathway. The selected concentrations of inhibitors inhibited internalization of fluorescein isothiocyanate–transferrin and fluorescein isothiocyanate–dextran, respectively (Supplemental Figure IVA). Using uPA-induced degradation of myocardin as a readout of successful endocytosis/nuclear translocation of uPAR, we observed that this process was not affected by chlorpromazine but was sensitive to amiloride (Figure 4C). Accordingly, uPA-dependent ubiquitination of myocardin was not influenced by cell treatment with chlorpromazine but was abolished by amiloride (Figure 4D). When stained immunocytochemically, VSMCs revealed colocalization of uPAR with early endosomal antigen in response to uPA but not in response to the amino-terminal fragment of uPA lacking the proteolytic activity (Figure 4E). Interestingly, uPAR also colocalized with PDGFRβ in endosomes (Figure 4F), and uPA-dependent myocardin degradation was sensitive to cell treatment with the PDGFRβ tyrosine kinase inhibitor AG 1295, as well as silencing of PDGFRβ (Figure 4G). Activation of PDGFRβ by ligand binding leads to receptor internalization and commonly to degradation by either lysosomal or proteasomal activity. The observation that the amino-terminal fragment of uPA, which can induce PDGFRβ-dependent signaling in VSMCs, failed to induce internalization of uPAR (Figure 4E), suggests that PDGFRβ-mediated signaling and uPAR-dependent myocardin degradation most probably represent different steps of uPA/uPAR-induced intracellular events in VSMCs.

uPAR Nuclear Import Is Mediated by SUMOylated RanGAP1

We next addressed the intracellular mechanism responsible for the uPA stimulated nuclear uPAR import. The small GTP-binding protein Ran is involved in the majority of nucleocytoplasmic transport pathways. In turn, Ran GTPase activating protein 1 (RanGAP1) activity regulates the activity of Ran. In vertebrate cells, this mechanism requires RanGAP1 modification by the small ubiquitin-like modifier (SUMO1). To analyze the role of RanGAP1 in uPAR nuclear translocation, we first addressed the question of whether or not uPA is able to initiate association of uPAR and RanGAP1 on cell stimulation. Indeed, we observed co-IP of both proteins in response to uPA in a time-dependent fashion (Figure 5A). Under these conditions, RanGAP1 underwent SUMOylation by SUMO1 (Figure 5B). Kinetics of the uPA-induced uPAR-RanGAP1 association and those of RanGAP1 SUMOylation suggest that this mechanism might mediate uPAR nuclear import. Thus, RanGAP1 SUMOylation reached maximum at 30 minutes of uPA stimulation; whereas its association with uPAR was observed at 45 to 60 minutes, and uPAR was found in the nucleus after 1 hour. The uPAR complex with PDGFRβ, which was formed rapidly after uPA stimulation, dissociated before uPAR came into interaction with RanGAP1 (Supplemental Figure IVB). We next analyzed the requirement of RanGAP1 for uPAR nuclear import in VSMC after RanGAP1 downregulation using small interfering RNA gene silencing (RanGAP1si) (Supplemental Figure IVC). RanGAP1 downregulation strongly inhibited uPAR nuclear translocation and uPA-induced myocardin degradation (Figure 5C–E). Collectively, these results indicate that uPA initiates RanGAP1 modification by SUMO1 and that SUMOylated RanGAP1 promotes uPAR nuclear import.

uPAR Mediates VSMC Phenotypic Changes After Vascular Remodeling In Vivo

To evaluate the in vivo pathophysiological relevance of our findings, we used a vascular injury animal model by performing carotid artery ligation in mice. After an early phase of inflammatory cell recruitment, medial VSMCs undergo switching from contractile to synthetic phenotype and rapidly proliferate and migrate toward the lumen, leading to extensive neointima formation in this model. uPAR-deficient mice and the corresponding wild-type control animals were used. No morphological differences were observed in sham carotid artery cross-sections of wild-type and uPAR−/− mice. Morphometric analysis of media thickness performed on stained frozen sections of uPAR−/−, and wild-type animals revealed significant differences between the mice as early as day 3. From day 3 to day 28, medial and neointima areas were further increased in wild-type mice, whereas uPAR−/− revealed much less vascular remodeling (Figure 6A–C).

TaqMan analysis performed 24 hours after carotid ligation demonstrated 20-fold upregulation of uPAR expression in ligated vessel of wild-type animals (Figure 6D). Increased content of uPAR in ligated vessel was accompanied by nuclear localization of uPAR in ligated vessel (Figure 6E). Accordingly, the expression of contractile proteins in ligated carotid arteries of wild-type animals was strongly downregulated 24 hours after vessel ligation. On the contrary, in the ligated carotid arteries of uPAR−/− mice, the decrease in contractile protein expression was far less pronounced (Figure 6F).

We further assessed the protein level of myocardin expression 24 hours after vessel ligation. Myocardin was decreased in wild-type mice but not in uPAR−/− mice (Figure 6G), suggesting that myocardin degradation in uPAR−/− mice might be impaired. Next, we assessed total 20S proteasomal activity in aortas of wild-type and uPAR−/− mice. Surprisingly, we observed that 20S proteasomal activity in uPAR−/− mice was much lower than in wild-type animals (Figure 6H).

Nuclear uPAR Serves as a Marker for Substrate Topography-Mediated VSMC Phenotype

Increasing evidence suggests that substrate topography, independent of its biochemical and biomechanical properties,

Figure 6 (Continued). Images were obtained using confocal scanning. Arrows in the right panel show nuclear localization of uPAR. Scale bar=10 μm. F, Expression of contractile proteins (smooth muscle α-actin [SMA], calponin, and SM22α) mRNA in control and ligated carotid artery of WT and uPAR−/− mice 24 hours after vessel ligation (n=5). G, Protein expression of myocardin in control and ligated carotid artery was assessed by Western blotting (n=4). H, 20S proteasomal activity was measured in homogenates of WT and uPAR−/− aortas (n=7). I, VSMCs cultivated on plane and structured ORMOCER substrate were uPA stimulated, fixed, and stained for myocardin and uPAR. Scale bar=10 μm.
Because the RanGAP/importin mechanism mediates nuclear SUMOylated RanGAP1 is required for uPAR nuclear transport.

We found that the glycosylphosphatidylinositol-anchored receptor uPAR can conduct nuclear signaling to interfere with the myocardin-controlled gene regulation of VSMC phenotypes in vitro and in vivo. Our results could have important functional implications because they point to the as yet unknown ability of uPAR to affect directly nuclear events of transcriptional regulation. We observed that uPAR is able to undergo a ligand-induced endocytosis and nuclear translocation. Our findings suggest that uPAR/myocardin interaction in the nucleus induces displacement of myocardin from SRF, thus repressing myocardin’s ability to activate VSMC gene expression and leading to ubiquitin-mediated myocardin degradation via proteasomal pathway. Chromatin immunoprecipitation analysis confirmed that uPA and uPAR indeed regulate SRF association with promoters of contractile genes in a proteasome-dependent manner. We investigated the phenomenon using biochemical and imaging approaches including FRET. We show that N-terminal domain of myocardin is required for interaction with uPAR.

Several groups recently described mechanisms related to myocardin regulation of smooth muscle and cardiac gene expression via interaction with SRF. A number of factors were identified that influence myocardin/SRF binding, including the steroid receptor coactivator 3 complex, which identifies a site of convergence for nuclear hormone receptor-mediated and VSMC-specific gene regulation. Our data add the signal-dependent interaction of uPAR with myocardin leading to myocardin degradation by the proteasome as a novel functional partner for cell differentiation machinery.

Several types of transmembrane receptors, such as heptahelical G-protein-coupled receptors and tyrosine kinase receptors, are translocated to the nucleus, where they have activities distinct from those expressed at the cell surface. However, nothing is known about any glycosylphosphatidylinositol-anchored cell surface proteins that participate in nuclear import. The receptors may be imported to the nucleus using classical basic nuclear localization signal (NLS) or because of association with NLS-containing carrier proteins.

Recently a uPAR-independent nuclear translocation of its ligand uPA via NLS sequence of nucleolin has been reported. We found that SUMOylated RanGAP1 is required for uPAR nuclear transport. Because the RanGAP/importin mechanism mediates nuclear localization of NLS-bearing receptors, the existence of uPAR domain functioning as a noncanonical NLS might be suggested.

The next question that must be addressed is the pathway of uPAR internalization coupled to its nuclear translocation. Aside from the canonical pathway of uPAR ligand-induced endocytosis, several alternative and uncommon mechanisms of uPAR internalization exist. We found that uPAR relies on PDGFRβ for the internalization step that seems to be clathrin independent but relies on vesicles trafficking. Clathrin-independent internalization, using macropinocytosis-like internalization pathways, was reported for receptor tyrosine kinases such as PDGFR. The relatively slow rate of uPAR internalization also speaks in favor of a macropinocytosis-like mechanism. Our earlier study showed that PDGFRβ serves as a transmembrane adaptor of uPAR in VSMCs. We showed that uPAR/PDGFRβ signaling does not require uPA proteolytic activity because it was fully activated by the amino-terminal fragment of uPA. Interestingly, we observed here that uPA proteolytic activity is indispensable for uPAR nuclear translocation. Most probably, rapid activation of intracellular signaling and the more gradual internalization and nuclear transport of uPAR represent 2 sequential steps of the process.

The generation of mice deficient in uPA and uPAR genes has demonstrated that the severity of vascular damage is highly dependent on the fibrinolytic system. We and others have shown that uPAR targeting could effectively inhibit vascular remodeling after injury. PDGFRβ deficiency, however, does not compromise embryonic development of mice. No histological abnormalities in arterial wall and other tissues of adult uPAR−/− mice were observed in the absence of challenging factors such as injury or infection. Together, these observations represent an interesting, not yet fully addressed feature of uPA/uPAR system.

Despite the obvious clinical relevance of these findings, the mechanisms responsible for the observed beneficial effect of uPAR deficiency on vascular remodeling remained unknown. Our results highlight the underlying molecular mechanism and show that phenotypic modulation of VSMCs in vivo in mouse carotid artery ligation model is also a uPAR-regulated phenomenon. Thus, in uPAR gene–deleted mice, decrease of contractile protein expression was strongly impaired, which resulted in attenuated neointima formation. Our injured-vessel model underscored the idea that the uPA/uPAR system is involved in regulation of activity of proteasomes linking the plasminolytic and proteasomal systems.

VSMCs continuously interact with tissues that possess topographical surface features because of the presence of proteins embedded in the vascular wall. Bioimplants intended to restore blood flow do not have micro- or nanoscale surface features. This state of affairs could underlie an undesired device-tissue response. Our data show that topographically treated VSMCs have a distinct differentiation profile. We found that whereas uPAR exhibited nuclear localization in synthetic VSMCs on nonstructured substrate, no nuclear uPAR was observed in cells cultured on ORMOCER. These data strongly suggest that engineered nanopothgraphical cues may influence VSMC behavior in a manner that could be relevant for vascular stents and other vascular applications. The data further indicate that the phenotypic state of VSMCs on structured
substrates involves a mechanism of uPAR nuclear transport, and therefore, uPAR promises to be a powerful tool to control tissue-bioimplantable device response.

Acknowledgments
We thank Petra Wuebbolt-Lehm and Birgit Habermeier for excellent technical assistance and F. C. Luft for editing the manuscript.

Sources of Funding
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Disclosures
Y. Kiyan received ERA-AGE FLARE grant financed in Germany by Bundesministerium für Bildung und Forschung (01 ET 0802).

References


Urokinase Receptor Associates With Myocardin to Control Vascular Smooth Muscle Cells Phenotype in Vascular Disease

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Supplement Material

Materials and methods

Antibodies and chemicals

Monoclonal (MAB807), polyclonal (AF807) anti-human uPAR antibodies, anti-rodent uPAR (MAB531) were from R&D Systems.

For detection of myocardin in western blotting after non-reducing electrophoresis monoclonal anti-myocardin antibodies (MAB4028) from R&D Systems was used. For immunoprecipitation of myocardin polyclonal anti-myocardin A-13 antibody from Santa Cruz Biotechnology was used. Polyclonal anti-myocardin M-16 antibody was used for immunocytochemical staining of fixed VSMC.

Polyclonal anti-RanGAP1 (N-19), polyclonal anti-SUMO1 (FL-101) antibodies, monoclonal anti-26S (p32), and monoclonal anti-GAPDH (6C5) antibody were from Santa Cruz Biotechnology. Monoclonal anti-SMA (clone 1A4) and anti-calponin (clone hCP), were from Sigma. Polyclonal anti-SM22α (ab14106) antibodies were from Abcam. Monoclonal anti-uPAR (3937) antibodies from America Diagnostica were used for immunoprecipitation. Anti-DDK monoclonal antibody was from Origene. Alexa 488-conjugated chicken anti-goat antibodies and Alexa 633 rabbit anti-mouse antibodies, Alexa 488- and Alexa 594-Zenone labeling kits were from Invitrogen Ltd. DraQ5™ DNA stain was from Biostatus Limited.

siRNA duplexes (uPAR, myocardin, RanGAP1, and uPA) were from Santa Cruz Biotechnology (Santa Cruz, CA). Myocardin pre-design chimera RNAi and control chimera RNAi were from Abnova GmbH (Heidelberg, Germany). Vector for expression of DDK-tagged human myocardin was purchased from Origene (RC229055).
The constructs to express luciferase under smooth muscle α actin and SM22α promoters were a kind gift of Dr. G. Owens, University of Virginia School of Medicine. Constructs to express GST-fusion mutants of myocardin were kindly given by Dr. Zhou.

Amiloride, chlorpromazine, AG1295, and other high grade chemicals were from Sigma. Proteasome inhibitor AM-114 was from Calbiochem. Human urokinase for VSMC stimulation was from Loxo.

**Cell culture and transfection**

Human primary VSMC were isolated from umbilical artery using explant technique in VascuLife SMC cell culture medium (CellSystems® Biotechnologie Vertrieb GmbH, St. Katharinen, Germany). The tissues were treated accordingly to local Ethics Commission requirements. After 1st passage the fibroblasts were removed from the culture by cell separation using monoclonal anti-fibroblasts antibodies (anti-CD90, Dianova GmbH, Hamburg, Germany) and magnetic Dynabeads® Goat anti-Mouse IgG (Invitrogen GmbH, Krlsruhe, Germany). VSMC were used between passages 2-4. Human urokinase for cell stimulation was purchased from Loxo and applied to cells at concentration of 10 nM. Murine urokinase was a kind gift of Dr S. Yarovoi, Dept. of Pathology and Laboratory Medicine, University of Pennsilvania.

Aortic VSMC from uPA-/- and corresponding WT control animals were isolated by enzymatic digestion as described and cultivated in DMEM containing 10% FCS.

Primary VSMC were transfected using Amaxa Nucleofector I (Lonza Group Ltd) and Human Primary Smooth Muscle Cells Basic Nucleofection Kit accordingly to manufacturer’s instructions. Lentiviral infection of mouse VSMC was performed as described.

For murine uPAR silencing, the target sites in mRNA for RNAi were determined using the siRNA Selection Server (http://jura.wi.mit.edu/bioc/siRNAext/home.php) and designed as
oligonucleotides encoding short hairpin RNAs (shRNAs). The following complimentary sequences were selected: Plaur 464 (CAGAAAGGAGCTTGAAGGATGAG); Plaur 561 (GTGAAAGGTCTGGTGTGATGGA).

Chromatin immunoprecipitation (ChIP)

ChIP analysis was performed using SimpleChIP™ Enzymatic Chromatin IP kit (Cell Signaling Technology). Anti-SRF antibody (G-20, Santa Cruz) was used for immunoprecipitation and then SRF association with SMA and SM MHC promoters was estimated using SYBR-Green RT-PCR. For positive control Histon H3 antibody and RPL30 primers were used (Cell Signaling Technology). Corresponding normal IgG were used as negative control. ChIP was performed overnight at 4°C with rotation. Input DNA was purified along with ChIP probes. Association with SMA and SM-MHC promoters was estimated using SYBR-Green RT-PCR. Sequence of primers used for ChIP is given in Supplementary Table 1. Fos primers were used to show SRF binding to irrelevant promoters.

Subcellular fractionation and biochemical methods

Subcellular fractionation was performed using Subcellular Protein Fractionation Kit (Pierce, Thermo Scientific). Stepwise extraction allowed isolating soluble cytoplasmic proteins; membrane (plasma, mitochondria and endoplasmic reticulum/golgi but not nuclear membrane) extract; soluble nuclear extract; and finally a second nuclear extraction with micrococcal nuclease is performed to release chromatin-bound nuclear proteins.
For co-immunoprecipitation study from the whole cell lysate, subconfluent and serum-starved VSMC were treated with 10 nM uPA at 37°C and lysed in modified RIPA lysis buffer (50 mM Tris-HCl, pH 7.4; 1% Triton X-100; 0.25% Na-deoxycholate; 150 mM NaCl; 1 mM EDTA) supplemented with 1mM PMSF; 1 µg/ml aprotinin; 1 µg/ml leupeptin; 1mM Na3VO4; 1mM NaF directly on culture dishes. Lysates were clarified by centrifugation at 12,800 rpm for 10 min and were used for western blotting or immunoprecipitation. Typically, 5 µg of antibodies were incubated with 600-1000 µg of cell lysate protein for 2 hrs, then protein A/G agarose was added and incubation proceeded for another hour. Then beads were washed 3 times with 500 µl PBS containing 1mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1mM Na3VO4, 1mM NaF, solubilized in Laemmly SDS sample buffer and then analyzed by Western blotting.

Co-immunoprecipitation of nuclear proteins was studied using Nuclear Complex Co-IP Kit (Active Motif). Nuclei lysis and recovery of nuclear proteins was performed in low-salt buffer protecting nuclear proteins complexes followed by digestion with proprietary enzymatic shearing cocktail that allows release of DNA-associated proteins. Distribution of cytosolic and nuclear marker proteins in nuclear and cytosolic extracts was controlled by western blotting. For non-specific binding control corresponding IgG were incubated with cell lysate and protein A/G agarose in all experiments.

20S proteasome activity assay

20S proteasome activity was measured fluorimetrically with the use of Assay kit from Cayman Chemical Company using SUC-LLVY-AMC as a substrate.

Biotinylation assay.

Subconfluent cells were washed with Ca2+/Mg2+-PBS, and acid-treated in 50 mM Glycine hydrochloride/100 mM NaCl, pH 3, for 3 minutes at 0°C (to remove endogenously bound
ligands). Then acid solution was quickly neutralized with a half volume of 0.5 M Hepes/100 mM NaCl, pH 7.4. Then, cells were incubated 40 min on ice in the presence of 2 mg/mL NHS-SS-biotin in PBS. Biotinylated cells were then washed with PBS and incubated at 37°C to allow internalization. The samples were then put back on ice, washed and treated with two successive reductions of 20 min on ice with a reducing solution containing 42 mM GSH, 75 mM NaCl, 1 mM EDTA, 1% BSA and 75 mM NaOH. In this way, only the internalized proteins remained biotinylated. Cells were then carefully washed in cold PBS, and lysed with RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1% deoxycolate, 0.1% SDS, 1% Triton X-100) for 30 min. on ice. uPAR was immunoprecipitated from lysates to analyze the amount of receptor internalized. Peroxidase reaction was developed using streptavidine-HRP. A sample that was labelled but not reduced was included as a positive control, and a sample that was labelled and reduced, prior to incubation at 37°C, was included as a negative control. For antibody stripping membrane was incubated in 25 mM glycine-HCL, pH 2, 1% SDS for 30 min at RT, then washed, blocked and used with antibodies.

Luciferase activity was measured using Promega ONE-Glo™ luciferase assay system accordingly to manufacturer’s instructions.

Luciferase assay

For luciferase assay VSMC were nucleofected with constructs expressing luciferase under smooth muscle α actin and SM22α promoters (a kind gift of Dr. G. Owens, University of Virginia School of Medicine). Where required, cells were co-nucleofected with luciferase constructs and siRNA. 24 hrs after nucleofection cells were stimulated with uPA for 3-5 hrs and luciferase activity was measured using Promega ONE-Glo™ luciferase assay system accordingly.
to manufacturer’s instructions.

Quantitative RT-PCR analysis of uPAR in human VSMC

Total RNA was isolated from VSMC using RNeasy miniprep kit (Quiagen, Hilden, Germany). RNA from ligated and sham operated mouse carotid artery was isolated using RNeasy fibrous tissue mini kit (Quiagen, Hilden, Germany). Real-time quantitative RT-PCR for was performed on a LightCycler® 480 Real-Time PCR System using LightCycler® 480 RNA Master Hydrolysis probes (Roche Diagnostics GmbH, Mannheim, Germany). The oligonucleotide sequence of primers and probes is listed in supplemental Table S1. The data are normalized to relative SMA/GAPDH (or calponin/GAPDH) expression of control si-nucleofected cells or unstimulated cells. The results shown are the mean from 3 independent experiments ±SEM.

Immunofluorescent confocal microscopy

For immunocytochemical staining, cells were cultured on glass coverslips. Cells were fixed with 2% paraformaldehyde in PBS for 15 min at 4°C, permeabilized with 0.1% Triton X-100 in PBS for 3 min at 4°C, and blocked overnight at 4°C in 1% bovine serum albumin in PBS. Cells were labelled with primary antibody (2 hrs at room temperature) and fluorescently labelled secondary antibodies (1 h at room temperature). As DNA stains either DraQ5 or DAPI was used. After staining, cells were embedded in Aqua-Poly/Mount mounting media (Polysciences Europe GmbH, Eppelheim, Germany). The fluorescence cell images were captured using a Leica TCS-SP2 AOBS confocal microscope (Leica Microsystems). All the cell images were taken with oil-immersed x63 objective, NA = 1.4. Images were recorded with detection wavelengths range for
Alexa 488 and Alexa 594 double staining to 505 – 550 nm and 605 – 675 nm, respectively. All the images were acquired with a resolution of 1024 x 1024 pixels.

Images intensity profiles along the lines indicated on the figures were created using ImageJ (ImageJ 1.42, http://rsbweb.nih.gov/ij/download.html) software. Each profile shown in Figure 3 is result of averaging of 16 profiles obtained from 16 adjacent image lines.

For video reconstruction of 3D nuclear clastosome structure serial images of VSMC stimulated with uPA for 2 hrs and stained for uPAR (Alexa594) and myocardin (Alexa 488) were taken. 36 serial images were used for video reconstruction. The size of the scanned area was 33.4x33.4 μm². The z-position step was 0.12 μm.

FRET analysis

Anti- uPAR monoclonal antibodies (American Diagnostica, 3937) were conjugated to Alexa 594 using Zenon mouse IgG labeling kits from Molecular Probes (Invirogen), anti-myocardin polyclonal antibodies (M-16, Santa Cruz) were labeled with Alexa 488 goat IgG Zenon labeling kit (Molecular Probes, Invitrogen) as recommended by the manufacturer. Antibodies (10 μg/ml anti-uPAR; 5μg/ml anti-myocardin) were applied to uPA-stimulated cells simultaneously. Isotypic IgG labeled with the same procedure gave no detectable immunostaining. Images were acquired using a Leica TCS SP2 AOBS Confocal Microscope. The objective employed was the HCX PL APO CS 63.0x1.40 OIL. Images were recorded in the donor and acceptor channels with detection wavelengths range set to 505 - 575 nm and 610 – 750 nm, respectively. All the images were acquired with resolution of 1024x1024 pixels. Prior any data processing, the background noise level was subtracted from the pixel values for all the images. The background noise levels were obtained for the donor and acceptor channels by
acquiring and averaging of 100 images in the each of channel with excitation lasers off and microscope settings the same as for images acquisition.

The images of cell were recorded in the donor and acceptor channels under the excitation by 488 nm radiation from Ar-laser. The obtained pixel values after noise level subtraction are denoted as D_{488} and A_{488}, respectively. Then the image of the same cell was recorded in the acceptor channel under the excitation by 594 nm radiation from HeNe-laser. The obtained pixel values after noise level subtraction is denoted as A_{594}.

Following 5, the A_{488} signal is a sum of net FRET signal and fluorescence signals produced by each of the individual fluorophores in the acceptor channel. The latter contributions from the Alexa 488 and Alexa 594 fluorophores were calculated and subtracted from A_{488} signal in order to obtain net FRET signal F = A_{488} – δ D_{488} – ε A_{594}, where δ is bleedthrough factor for Alexa 488, and ε is bleedthrough factor for Alexa 594. Images of cells single-stained with Alexa 488- and single-stained with Alexa 594 conjugated antibodies were taken to calculate the bleedthrough factors δ and ε as described by 5. All microscope settings were kept unchanged while all images were acquired. The images acquisition sequence was the same for each cell. Finally, normalized FRET signal was calculated for each pixel in accordance with equation: \( F_N = \frac{F}{A_{594}} = \frac{A_{488}}{A_{594}} – δ \frac{D_{488}}{A_{594}} – ε \). This normalization allows to correct for the acceptor fluorophore concentration. The distribution of the normalized FRET signal (F_N) over cell was shown by color coding of the F_N signal at the cell pixels.

*Mouse carotid artery ligation model*

Animal experiments were approved by the regulatory board for animal welfare of the state of Niedersachsen, Germany. Male uPAR\(^{-/-}\) mice and uPAR\(^{+/-}\) mice as controls (all on
C57/BL6 background, age 10-12 weeks) were used for experiments. The left common carotid artery was ligated with a 6-0 suture proximal to the carotid bifurcation. Whole ligated carotid arteries were used to extract RNA for Taqman analysis, the contralateral carotid served as control. Whole necks were harvested for morphometrical analysis and immunostaining.

Morphometric analysis

At day 3 and day 28 after carotid artery ligation, the animals were euthanized by i.p. injection of ketamine/xylazine. Mouse tissue was perfusion fixed and the whole neck cryopreserved and embedded as described previously. Five cryosections (10 um thick) at 3-4 mm distal to the ligation site were obtained in each animal. The 4 different areas (lumen, intima, media and total vascular area) were measured in H&E stained sections using analyzing software (ImageJ 1.42, http://rsbweb.nih.gov/ij/download.html) as previously described with minor modifications. In brief, the areas surrounded by the luminal surface, internal elastic lamina (IEL), and external elastic lamina (EEL) were determined. The intimal area was determined by subtraction of the luminal area from the area defined by the IEL, and the medial area was calculated by subtraction of the area defined by the IEL from the area defined by the EEL. A mean value among 5 sections was used for analysis.

Immunostaining of frozen sections

Frozen sections were washed with PBS (with Ca++,Mg++) at RT for 5 min and block with 5% goat serum in PBS with Ca,Mg and 0.1% NP40 for 2 hrs at RT. The sections were incubated with anti-uPAR (M-17) antibody (Santa Cruz) in blocking solution overnight. On the next day the slides were washed 3x 15’ with PBS, incubated with chicken- anti goat Alexa 488 antibody
for 1 h at RT. Cells nuclei were labeled with DraQ5 and slides were mounted using Aqua Poly/Mount medium (Polysciences Europe GmbH, Eppelheim, Germany). Images were acquired using a Leica TCS SP2 AOBS Confocal Microscope. The objective employed was the HCX PL APO CS 63.0x OIL.

For myocardin analysis ligated and sham operated arteries of wild-type and uPAR-/- mice were dissected, washed from blood and homogenized in RIPA lysis buffer using glass pestle tissue grinder. Tissue lysates were used for western blotting.

Microstructured cell substrates fabrication

Microstructured cell substrates were fabricated from silicate-based inorganic-organic hybrid polymer ORMOCER (Microresist GmbH) by two-photon polymerization (2PP) with femtosecond pulses. For structuring, glass substrate was covered with a layer of liquid non-polymerized ORMOCER containing 1.8wt% of photoinitiator Irgacure369. ORMOCER gratings were fabricated by photopolymerization using Ti:Sapphire femtosecond laser. After laser patterning by 2PP, the non-polymerized ORMOCER was washed out by 4-methyl-2-pentanone. For the experiments ORMOCER gratings with cell size of 30 µm and 7.2 µm depth were used.

Statistical methods

Data were analyzed for statistical significance using the two-tailed Student’s t test for independent samples (OriginPro 8 SRO). Data are presented as mean ± SEM. Differences were considered statistically significant at a value of p<0.05.
**Supplemental Table SI.** Nucleotide sequence of primers used for TaqMan RT-PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td><strong>Myocardin</strong></td>
<td>Sense</td>
<td>5'-GACAGTAAAGAACCACCCACAAA-3'</td>
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<tr>
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<td>Antisense</td>
<td>CAG GGC TAC AGG GAAAGG CCG TGG AAA CCT G</td>
</tr>
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Supplemental Figures.

Figure SI.
Figure SII.
Figure SIII.
Figure SIV.
Supplemental Movie legend.

3D reconstruction of clastosome-containing cell nuclei stained for uPAR (Alexa 594) and myocardin (Alexa 488). Video is obtained from serial confocal images of uPA-stimulated VSMC fixed and stained as described in the methods section. The video shows co-localization of proteins in clastosome-like structures, with uPAR being localized in the core of the structure. 36 serial images were used for video reconstruction. The size of the scanned area was 33.4x33.4 µm². The z-position step was 0.12 µm.

Supplemental Figure legends.

Figure SI. A. uPAR expression downregulation in human VSMC. The expression of uPAR was quantified by TaqMan. GAPDH expression was used for quantification. B. uPA expression downregulation in human VSMC. The expression of uPA was quantified by TaqMan. GAPDH expression was used for quantification. C. Left. Myocardin expression in THP-1, U937 and human umbilical artery VSMC of passage 2 (two left lines) was assessed by western blotting using monoclonal anti-Myocardin antibody (R&D systems). Right upper panel. Monoclonal anti-myocardin antibody (R&D systems) detect myocardin expression in human VSMC cell lysates linearly. Right bottom panel Polyclonal anti-Myocardin antibody (A-13, Santa Cruz Biotechnology) immunoprecipitate myocardin from lysates of VSMC. D. Myocardin si was quantified by TaqMan and western blotting. The lower panel shows quantification of western blotting data. E. Polyclonal anti-Myocardin antibody (M-16, Santa Cruz Biotechnology) was used for staining of fixed VSMC. Application of the blocking peptide for the antibody prevents the myocardin nuclear staining. Myocardin si -nucleofected VSMC also show significantly less staining. F. Expression of SMA and calponin after myocardin si assessed by TaqMan. G.Left panel. Myocardin mRNA expression after uPARsi and uPA human VSMC stimulation. Right panel. Myocardin mRNA expression in WT and uPAR-/- mouse VSMC stimulated with uPA assessed by TaqMan. H. Myocardin expression after human VSMC nucleofection for overexpression of DDK-Myocardin assessed by TaqMan. I. Myocardin-overexpressing and vector-nucleofected VSMC were stained using anti-DDK antibody to show nuclear localization of overexpressed DDK-Myocardin. J. Distribution of cytoplasmic and nuclear markers in cytosol and nuclear extract obtained by Nuclear co-IP kit.

Figure SII. Single uPAR and myocardin stainings used for image profiling showed in Fig. 3Aand VSMC stimulated with uPA for 30 min. B. VSMC stimulated with uPA for 1h were fixed and stained for uPAR (Alexa 594) and myocardin (Alexa 488). DraQ5 was used as nuclear stain. C. Nuclear localization of uPAR (Alexa 594) in cell stimulated with uPA for 1h was analyzed by confocal scanning at different horizontal planes. DAPI was used as nuclear stain. Focal adhesion localization of uPAR is visible at the basal plane close to the substrate. Clear nuclear localization of uPAR was observed at the plane through the middle of the nucleus.
**Figure SIII.** A. Mouse aortic VSMC were stimulated with mouse uPA, then fixed and stained for murine uPAR. B. Distribution of marker proteins in cytosol, membrane extract, soluble nuclear extract and chromatin-bound nuclear extract obtained using Subcellular Fractionation Kit. C. Kinetics of uPAR and myocardin redistribution to soluble nuclear extract of human VAMS stimulated with uPA for indicated time.

**Figure SIV.** A. Inhibition of endocytosis. VSMC were treated with Amiloride ar chlorpromazine for 30 min at 37°C, then FITC-dextran was added and endocytosis was allowed for 40 min. Then cells were fixed and mounted. In case of Transferrin (Tf) endocytosis, cells were allowed to absorb Tf for 20 min on ice, then were washed with ice-cold PBS and placed at 37°C to allow endocytosis. After 20 min cells were fixed and mounted. B. Human VSMC were stimulated with uPA for indicated time. Whole cell lysate was used to immunoprecipitate uPAR. PDGFR and RanGAP1 were detected sequentially by western blottin to dissect kinetics of their interaction with uPAR. C. RanGAP1si in nucleofected VSMC was analysed by western blotting. D. SEM image of microstructured organically modified ceramics (ORMOCER®) substrate. E. VSMC cultivated on plane and structured ORMOCER substrate were fixed and stained for SMA and phalloidin.
Supplemental references


