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Fibulin-5 binds urokinase-type plasminogen activator and mediates urokinase-stimulated β 1-integrin-dependent cell migration

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Abstract

uPA (urokinase-type plasminogen activator) stimulates cell migration through multiple pathways, including formation of plasmin and extracellular metalloproteinases, and binding to the uPAR (uPA receptor; also known as CD87), integrins and LRP1 (low-density lipoprotein receptor-related protein 1) which activate intracellular signalling pathways. In the present paper we report

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AUTHOR CONTRIBUTION

Alexander Kapustin and Victoria Stepanova designed the study, performed the experiments and wrote the paper. Natalia Aniol performed the MEF Transwell and wound healing migration assays, adhesion and proliferation assays, and performed data analysis. Douglas Cines helped to design the experiments, analysed the results and helped to write the paper. Alexei Poliakov performed the cell motility experiments and helped to write the paper. Sergei Yarovoi expressed and purified mouse uPA. Tatiana Lebedeva performed experiments shown in Figures 3 and 5, propagated and immortalized MEFs. Robin Wait performed the mass spectrometric analysis and prepared Supplementary Table S1. Grigory Ryzhakov carried out the fibulin-5 cloning and performed fibulin-5-binding assays. Yelena Parfyonova discussed the results and wrote the paper. Robert Beabealashvili, Yaroslav Gursky and Mikhail Minashkin cloned and expressed human uPA and its mutant forms. Hiromi Yanagisawa developed the anti-(fibulin-5) antibody and the fibulin-5-knockout/wild-type MEFs and the *Fbln5*^{RGE/RGE} MEFs. Alexander Vorotnikov contributed his expertise in cell motility and adhesion, and participated in writing the paper. Alex Bobik provided scientific guidance and wrote the paper. Vsevolod Tkachuk conceived the study, provided scientific guidance and institutional support, and wrote the paper. All authors contributed to editing the paper, and approved the final version of the paper.

that uPA-mediated cell migration requires an interaction with fibulin-5. uPA stimulates migration of wild-type MEFs (mouse embryonic fibroblasts) (*Fbln5*^{+/+} MEFs), but has no effect on fibulin-5-deficient (*Fbln5*^{-/-}) MEFs. Migration of MEFs in response to uPA requires an interaction of fibulin-5 with integrins, as MEFs expressing a mutant fibulin-5 incapable of binding integrins (*Fbln*^{RGE/RGE} MEFs) do not migrate in response to uPA. Moreover, a blocking anti-(human β 1-integrin) antibody inhibited the migration of PSMCs (pulmonary arterial smooth muscle cells) in response to uPA. Binding of uPA to fibulin-5 generates plasmin, which excises the integrin-binding N-terminal cbEGF (Ca²⁺-binding epidermal growth factor)-like domain, leading to loss of β 1-integrin binding. We suggest that uPA promotes cell migration by binding to fibulin-5, initiating its cleavage by plasmin, which leads to its dissociation from β 1-integrin and thereby unblocks the capacity of integrin to facilitate cell motility.

Keywords

fibroblast; fibulin-5; migration; urokinase; vascular pathology

INTRODUCTION

uPA (urokinase-type plasminogen activator) and its receptor (uPAR) have been implicated in diverse physiological and pathological processes involving cell migration through fibrin and subcellular matrices, e.g. angiogenesis, wound repair, inflammation, immunity and tumour metastases [1–3]. These overlapping biological activities involve three properties of the uPA–uPAR system: the proteolytic activity of uPA, the promotion of cell adhesion by uPA–uPAR, and the initiation of signal transduction leading to cell proliferation and migration. uPA is secreted as a 411 amino acid single-chain proenzyme (termed scuPA) composed of an N-terminal GFD (growth factor-like domain) (amino acids 1–43) which binds to uPAR [4], a kringle domain (amino acids 44–135) and a C-terminal serine protease domain (amino acids 136–411). scuPA has little or no intrinsic enzymatic activity [5,6]. scuPA is activated upon cleavage by plasmin at Lys¹⁵⁸–Iso¹⁵⁹ to generate a two-chain molecule (termed tcuPA) which converts plasminogen into plasmin [7] and cleaves several other substrates, such as VEGF (vascular endothelial growth factor) and HGF (hepatocyte growth factor) [8,9]. Plasmin, in turn, degrades the extracellular matrix and stimulates the local release of growth factors promoting cell adhesion, migration and proliferation [10–12].

uPA-knockout mice (*Plau*^{-/-}), but not uPAR-knockout mice (*Plaur*^{-/-}), exhibit defects in fibrinolysis [13,14], neointima formation [15], hypoxia-induced hypertrophic remodelling of the pulmonary vasculature, and dissemination of certain tumours [16], among other phenotypes (see [17] for a review). uPA/uPAR expression in atherosclerotic human arteries is elevated ~ 2–5-fold above normal [18–20]. Our previous data demonstrated that uPA mediates neointima formation after injury to the carotid artery in rats; uPA catalytic activity, but not binding to uPAR, is essential for induction of neointima formation in this model [21]. Previous studies have demonstrated that uPA-induced cell migration may not exclusively rely on uPA–uPAR interactions. Plasminogen activation [22] or uPA interactions, as for other partners including LRP1 (low-density lipoprotein receptor-related protein 1) and integrins, can also stimulate cell migration [23,24]. Phenotypic differences

between *Plau*^{-/-}, *Plaur*^{-/-} and tPA (tissue plasminogen activator)-knockout mice (*Plat*^{-/-}) provide a compelling rationale for identification of novel receptors and pathways downstream of uPA involved in vascular remodelling and tissue repair, where cell migration plays an important role [25–27].

In the present paper we report that uPA interacts with fibulin-5, an integrin-binding extracellular matrix protein with an RGD motif. We demonstrate that uPA promotes cell migration by binding to fibulin-5 and promoting its cleavage. Proteolysed fibulin-5 dissociates from β 1-integrin, and therefore unblocks its capacity to promote cell migration.

MATERIALS AND METHODS

Materials

DMEM (Dulbecco's modified Eagle's medium), DPBS (Dulbecco's PBS), trypsin/EDTA and antibiotics were from Gibco (Invitrogen). Lipofectamine™ 2000, Protein A-agarose, the T7/CT TOPO TA kit, pCMV/cyto/Myc and pCEP4 expression vectors, the *Drosophila* S2 expression system, the tyramide signal amplification system, HRP (horseradish peroxidase)-conjugated anti-V5 antibody and electrophoresis reagents were from Invitrogen. Eight-well chamber slides were from Nalge Nunc International and Stripwell flat-bottomed 96-well plates were from Corning. FBS (fetal bovine serum) was from HyClone, hygromycin B was from Roche and Pfu polymerase was from Stratagene. The anti-c-Myc antibody-agarose conjugate, cation-exchange SP-Trisacryl M beads, protease inhibitor cocktail for mammalian cells, CHAPS, Triton X-114 and Triton X-100 were from Sigma. CNBr-activated Fast-Flow Sepharose 4 was from Amersham Bioscience and Ni-NTA (Ni²⁺ - nitrilotriacetate) agarose, the RNeasy Mini Kit and DNase were from Qiagen. Aprotinin (Trasylol) was from Bayer, and Iodo-Gen, sulfosuccinimidyl-6-(biotinamido)hexanoate, HRP-conjugated neutravidin, HRP-conjugated goat anti-mouse Ig and SuperSignal West Pico chemiluminescent substrate were from Pierce. Amicon Ultra-75 centrifugal filters and mouse monoclonal anti-(β 1-integrin) antibodies were from Millipore. HRP-conjugated streptavidin (ABC kit, Vector Laboratories), the Bradford assay and semi-dry blotter were from Bio-Rad Laboratories. Microchemotaxis chambers were from Neuro Probe. Diff-Quick dye was from Dade Behring. NcoI and DNA polymerase I (Klenow) were from New England Biolabs, and XhoI was from Fermentas. The human SMC (smooth muscle cell) cDNA library was provided by Dr Natalya Kalinina (MV Lomonosov Moscow State University, Moscow, Russia). Rabbit anti-c-Myc, rabbit polyclonal anti-fibulin-5 and mouse anti-IgG antibodies were from Santa Cruz Biotechnology. The rabbit anti-polyhistidine antibody was from eBioscience, non-specific mouse and rabbit anti-IgG was from Dako, and anti-rabbit antibody and Vectashield mounting medium were from VectorLabs. Alexa Fluor® 488-conjugated goat anti-rabbit and anti-mouse IgG, and Alexa Fluor® 555-conjugated goat anti-mouse IgG and ProLong Gold mounting medium were from Molecular Probes. Mouse monoclonal antibodies against human urokinase B-chain (#3689) and human uPAR (#3937) were from American Diagnostica. An anti-(β 1-integrin) polyclonal antibody was from Epitomics and an anti-(fibulin-5) mouse monoclonal antibody was from Proteintech. Imaging software was from Scion. Affinity-purified rabbit antibody (BSYN

1923) against the rat fibulin-5 peptide YRGPYSNPYSTSYSGPYPAAPP was generated as described previously [28].

Cell lines and culture

MEFs (mouse embryonic fibroblasts) were isolated from *Fbln5*^{-/-} mice and their littermate controls as described previously [28]. *Fbln5*^{-/-} and *Fbln5*^{+/+} MEFs were spontaneously immortalized following the NIH 3T3 protocol [29]. HEK (human embryonic kidney)-293 cells, CHO (Chinese-hamster ovary) cells and MEFs were grown in DMEM containing 10 % (v/v) FBS, glutamine (5 mM), penicillin (100 i.u./ml) and streptomycin (100 µg/ml) at 37 °C under 5 % CO₂. PSMCs (pulmonary artery SMCs) were obtained from Cascade Biologics (Invitrogen) and cultured in Medium 231 supplemented with SMGS (smooth muscle growth supplement) (Cascade Biologics) at 37 °C under 5 % CO₂.

Recombinant urokinase

Recombinant wild-type human scuPA, scuPA lacking the N-terminal 1–43 amino acid residues comprising the GFD (uPA- GFD), low-molecular-mass uPA (uPA-LMW) and the N-terminal 1–166 amino acid residues fragment of uPA (uPA-ATF) were expressed in *Escherichia coli* and purified as described previously [30,31]. Mouse wild-type uPA was expressed in S2 cells and purified using cation-exchange SP-Trisacryl M beads (Sigma) as described previously [32].

Fibulin-5-expressing vectors pCMV-FBLN5 vector

Human fibulin-5 was PCR-amplified from a human SMC cDNA library with the following primers: 5'-CCAGGAATAAAAAGGATACTCACTGTTACC-3' and 5'-AAACTCGAGGAATGGGTACTGCGAC-3'. This procedure removed the ATG start codon in the 5' untranslated region and introduced an XhoI site in-frame in place of the termination codon. The mammalian expression vector pCMV/cyto/Myc was digested with NcoI, blunt-ended with DNA polymerase I (Klenow) and digested with XhoI. The PCR fragment was cloned into pCMV/cyto/Myc to yield the pCMV-FBLN5 vector, which encodes full-length human fibulin-5 with a c-Myc epitope at the C-terminus. The identity of the cloned gene to human *FBLN5* (GenBank® accession number NM_006329) was confirmed by direct and reverse sequencing.

pCEP4-FBLN5 vector—A C-terminal V5-His₆-tagged human fibulin-5 fusion construct was generated with the T7/CT TOPO TA kit according to the manufacturer's protocol. cDNA encoding full-length human fibulin-5 was amplified using the pCMV-FBLN5 vector as a template with primers which introduced a KpnI site and Kozak sequence in the 5' untranslated region and removed the termination codon. The sequences of the direct and reverse primers (with the restriction site underlined) are: 5'-CTATGGTACCGCCACCATGCCAGGAATAAAAAGGATACT-3' and 5'-GAATGGGTACTGCGACACATATATC-3' respectively. The PCR product was ligated into the T7/CT TOPO vector producing an intermediate plasmid TOPO-FBLN5. The full-length fibulin-5 cDNA followed by a sequence encoding V5 and His₆ tags at the 3' end was amplified by PCR using primers that introduce a KpnI site in the 5' untranslated region and a NotI site in the 3' untranslated region (direct, 5'-

CTATGGTACCGCCACCATGCCAGGAATAAAAAGGATACT-3'; and reverse, 5'-TATTGCGGCCGCTCAATGGTGATGGTGATG-3') and the TOPO-FBLN5 vector as a template. The PCR product was digested with KpnI and NotI and cloned into the mammalian expression vector pCEP4.

pWPXL-ncFBLN5 vector—QuikChange[®] mutagenesis to introduce an R77A mutation [33] into the *FBLN5* sequence was performed using the pCMV-FBLN5 vector as a template and 5'-CGGACAAACCCTGTGTATGCAGGGCCCTACTCGAACCCCT-3' and 5'-AGGGGTTTCGAGTAGGGCCCTGCATACACAGGGTTTGTCCG-3' primers using the QuikChange[®] Site-Directed Mutagenesis kit (Stratagene) to obtain the pCMV-ncFBLN5 vector (the sequence encoding alanine is underlined) [which encodes nc (non-cleavable) fibulin-5]. The full-length nc-fibulin-5 cDNA followed by a sequence encoding a c-Myc tag at the 3' end was amplified by PCR using the pCMV-ncFBLN5 vector as a template and using the primers that introduce an MluI site in the 5' untranslated region and an SpeI site in the 3' untranslated region, and then cloned into the pWPXL vector (Addgene and D. Trono laboratory, EPFL-SV-GHI-LVG, Station 19, CH-1015, Lausanne, Switzerland) and digested with MluI and SpeI. Lentivirus production using empty pWPXL and pWPXL-ncFBLN5 as transfer vectors was performed as described previously [34].

Generation of fibulin-5-expressing cell lines

Transient transfections of CHO and HEK-293 cells were performed using Lipofectamine[™] 2000 according to the manufacturer's protocol. To generate stable cell lines, HEK-293 cells were transfected with either pCMV-FBLN5 or pCEP4-FBLN5. At 48 h post-transfection, culture medium was changed to complete medium supplemented with 150 µg/ml Geneticin or 200 µg/ml hygromycin B respectively, and antibiotic-resistant clones were selected by serial dilution.

Purification of the fibulin-5 fusion protein

Recombinant human V5-His₆-tagged fibulin-5 (fibulin-5-V5-His) was purified using metal-chelate chromatography with a Ni-NTA column. The purity (~ 95 %) of the recombinant fibulin-5-V5-His protein was confirmed by SDS/PAGE and Western blotting (results not shown). Briefly, HEK-293 cells stably transfected with pCEP4-FBLN5 vector were grown in DMEM containing 10 % (v/v) FBS until they reached confluence. The cells were then switched to serum-free medium for 48 h at 37 °C. Cell culture medium was collected and clarified by centrifugation at 5000 g for 10 min. The supernatants were supplemented with PMSF (1 mM final concentration) and protease inhibitor cocktail for mammalian tissues and then applied to Ni-NTA agarose equilibrated with 50 mM NaH₂PO₄ (pH 8.0) buffer containing 0.3 M NaCl. Fibulin-5-V5-His was eluted from the Ni-NTA column with 50 mM NaH₂PO₄ (pH 8.0) buffer containing 0.3 M NaCl and 50 mM imidazole. Purified protein was concentrated and transferred to PBS using Amicon Ultra-75 centrifugal filters.

Purification and mass spectrometric analysis of uPA-binding proteins from human aorta

Thoracic aorta samples were obtained post-mortem (autopsies performed within 1–5 h of death) from individuals (aged 15–55 years) who died from different accidental causes, with appropriate ethical approval from the Cardiology Research Center Animal Care and Human

Experimentation Committee. Cell membrane fractions from human aortae were isolated as described previously [35]. All procedures were performed at 4 °C. Briefly, human aortae (20 g) were dissected and the medium was homogenized in 100 ml of buffer A [20 mM Mops (pH 7.4) containing 250 mM sucrose and 2 mM PMSF] and centrifuged at 3000 *g* for 10 min. Supernatants were centrifuged at 45 000 rev./min (rotor type 45 Ti) for 1 h. Pellets were solubilized in 20 ml of lysis buffer [0.1 M Tris/HCl (pH 8.1) containing 1 % (v/v) Triton X-114, 5 mM CHAPS, 5 mM EDTA, 100 k-units/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin and 0.5 mM PMSF. uPA- GFD- or BSA-sepharose, prepared as described previously [31] (1 ml), was added to the lysates for 2 h at 4 °C. The beads were centrifuged and washed in lysis buffer. The bound proteins were eluted with an equal volume of 2× non-reduced Laemmli buffer and were separated using SDS/PAGE (7 % gels). Gels were stained with either silver or with 0.2 % Coomassie Brilliant Blue R-250. Protein bands were excised and digested in-gel with trypsin using an Investigator Progest robot (Genomic Solutions) as described previously [36]. Samples were analysed by HPLC coupled to ESI (electrospray ionization)-MS/MS (tandem MS). HPLC was carried out on a CapLC liquid-chromatography system (Waters). Aliquots (6 µl) of peptide mixtures were injected on to a Pepmap C18 column (300 µm×0.5 cm; LC Packings) and eluted with an acetonitrile/0.1 % formic acid gradient to the nanoelectrospray source of a Q-TOF (quadrupole–time-of-flight) spectrometer (Micromass) at a flow rate of 1 µl/min. The spray voltage was set to 3500 V and data-dependent MS/MS acquisitions were performed on precursor peptides with charge states of 2, 3 or 4 over a survey mass range 440–1400 using argon collision gas. The recorded product ion spectra were transformed into a singly charged *m/z* axis using a maximum entropy method (MaxEnt3, Waters), and centroided *pkl* (peaklist) files were extracted using the MassLynx routine peptide auto (Waters). Proteins were identified by correlation of uninterpreted spectra to entries in SwissProt (Release 2010_04: 516081 entries) using a local installation of Mascot (version 2.2; <http://www.matrixscience.com>). MS/MS ion searches specified up to two missed cleavages per peptide, a precursor mass tolerance of ±100 p.p.m. and a fragment ion mass tolerance of ±0.5 Da. Carbamidomethylation of cysteine residues and methionine oxidation were specified as fixed and variable modifications respectively. Criteria for MS/MS-based peptide and protein identifications were validated using Scaffold (Proteome Software, version 3.01). Peptide identifications were accepted if they could be established at greater than 95.0 % probability as specified by the Peptide Prophet algorithm [37]. Protein identifications were accepted if established at greater than 99.0 % probability and contained at least two matched peptides. Protein probabilities were assigned by the Protein Prophet algorithm [38].

Co-immunoprecipitation, immunoblotting and ligand blotting

Aliquots of the cell culture media containing fibulin-5–c-Myc were incubated with 6 nM biotinylated scuPA for 1 h at room temperature (20 °C). Affinity matrices were prepared by incubating rabbit polyclonal anti-c-Myc antibodies or control rabbit IgG with Protein G–agarose for 1 h and blocking by incubation with PBS containing 3 % BSA for 1 h at room temperature. The complexes between scuPA and fibulin-5–c-Myc were precipitated using c-Myc polyclonal antibodies immobilized on Protein G–agarose. Immobilized rabbit IgG was used as the negative control. Bound proteins were eluted with non-reducing 2× Laemmli buffer and analysed by Western blotting. Biotinylated scuPA was probed with HRP-

conjugated neutravidin, and fibulin-5–c-Myc was detected using a c-Myc monoclonal antibody and HRP-conjugated anti-mouse secondary antibody.

To study the interaction of uPA and fibulin-5 by ligand blotting, cell culture medium from CHO cells transiently transfected with pCMV-FBLN5 or control CHO cells transfected with pCMV-GFP (GFP is green fluorescent protein) were incubated with scuPA–Sepharose for 1 h at room temperature. Bound proteins were eluted with 2× Laemmli buffer in the absence of reducing agents, subjected to SDS/PAGE and transferred on to PVDF membranes, which were blocked with PBS containing 0.05 % Tween 20 and 0.5 % gelatin. Fibulin-5–c-Myc was visualized either by sequentially incubating the membranes with 12 nM biotinylated wild-type scuPA and HRP-conjugated neutravidin, or with a c-Myc polyclonal antibody and an HRP-conjugated anti-rabbit antibody.

Immunocytochemistry

Fixed cells were incubated with either anti-uPA monoclonal or polyclonal antibodies (American Diagnostica) or anti-(β 1-integrin) polyclonal antibodies (Epitomics), anti-(fibulin-5) mouse monoclonal antibodies (Proteintech Group) or rabbit polyclonal anti-(fibulin-5) antibodies (Santa Cruz Biotechnology) followed by incubation with Alexa Fluor® 555-conjugated goat anti-mouse antibody or Alexa Fluor® 488-conjugated goat anti-rabbit antibody (Molecular Probes). Stained cells were mounted in ProLong Gold antifade reagents and analysed using a Zeiss LSM 510 laser-scanning confocal microscope (Carl Zeiss). To stain focal-adhesion contacts, MEFs were seeded on to the coverslips and incubated in DMEM containing 10 % (v/v) FBS until they reached 70–80 % confluence. Cells were washed with PBS, fixed in 4 % formaldehyde for 20 min and washed again twice with PBS. The cells were then permeabilized with 0.2 % Triton X-100 in PBS, washed with PBS, and incubated with blocking buffer (PBS containing 10 % goat serum and 1 % BSA) for 1 h. Cells were incubated with anti-vinculin primary antibody for 16 h followed by incubation with Alexa Fluor® 488-conjugated goat anti-mouse antibody. Cell nuclei were visualized with DAPI (4',6-diamidino-2-phenylindole) and analysed using a Leica AF 6000LX microscope.

Solid-phase assay

The affinity of uPA for fibulin-5 was assessed using a solid-phase binding assay. Plates (96-well) were coated with 5 μ g/ml recombinant fibulin-5–V5–His or BSA in DPBS supplemented with 10 mM CaCl₂ and 5 mM MgCl₂. Non-specific binding was blocked with PBS containing 1 % BSA and 0.05 % Tween 20). uPA–GFD was iodinated using Na¹²⁵I [39]. ¹²⁵I-labelled uPA–GFD (0.5–400 nM) was added in the binding buffer (PBS containing 0.1 % BSA and 0.05 % Tween 20) for 1 h at room temperature, and unbound ligand was removed by washing with binding buffer; bound radioactivity was measured in a γ -counter. Non-specific binding was measured in the presence of a 100-fold molar excess of unlabelled uPA–GFD. Specific binding was calculated as a difference between total and non-specific binding. All assays were performed in triplicate in two separate experiments. Data are presented as the molar amount of ¹²⁵I-labelled uPA–GFD specifically bound per well. The K_d was calculated using GraphPad Prism 4.0 (GraphPad Software). To study fibulin-5 binding to tcuPA, 96-well plates were coated with 35 μ g/ml recombinant tcuPA or

BSA in DPBS, supplemented with 10 mM CaCl₂ and 5 mM MgCl₂, blocked with DPBS supplemented with 1 % BSA and incubated with fibulin-5-V5-His (10–500 nM) for 1 h. After washing with DPBS supplemented with 1 % BSA, bound fibulin-5-V5-His was detected using HRP-conjugated anti-V5 antibody, followed by development with 3,3',5,5'-tetramethylbenzidine. The reaction was terminated by the addition of 1 mM H₂SO₄, and absorbance at 450 nm was measured using a microplate reader.

Cell adhesion

MEFs were incubated in DMEM containing 0.5 % FBS for 16 h, detached by brief treatment with 0.25 % trypsin, washed and immediately resuspended in DMEM containing 0.5 % FBS. Cells were then seeded in triplicate into 96-well plates (1×10⁴ cells/well) pre-coated with collagen type 1. After incubation in the presence or absence of mouse scuPA (25 nM), the cells were washed with PBS, fixed in 3.7 % paraformaldehyde and stained with Diff-Quick. Attached cells were counted visually in five microscopic fields in each well. The mean number of cells per field of view was determined from three independent experiments.

Transwell migration assay

Cell migration was measured in the 6.5 mm Transwell assay (Costar) using 0.8 μm pore-size membranes pre-coated with collagen type 1 for 1 h at 37 °C. MEFs were incubated in DMEM containing 0.5 % FBS for 16 h, trypsinized and re-suspended in DMEM containing 0.5 % FBS. Cells (10³ per well) were added in DMEM into the upper chamber. DMEM containing 0.5 % FBS or DMEM containing 0.5 % FBS and 25 nM scuPA was added to the lower chambers. The Transwells were incubated at 37 °C and 5 % CO₂ for 4.5 h, fixed and stained using Diff-Quick. Cells on the upper surface of the membrane were removed using a cotton swab and the cells that migrated to the lower side of the membrane were counted. The experiments were performed four times in duplicate.

Scratch assay

Cells were seeded in 12-well plates in DMEM containing 10 % (v/v) FBS and grown to 80 % confluence. Cells were then incubated in DMEM supplemented with 0.5 % BSA for 16 h, and scratched with a P10 pipette tip. After washing with DMEM, the cells were supplied with DMEM containing 0.5 % FBS or 0.5 % FBS and 25 nM mouse scuPA. Plates were transferred on to the microscopic stage of a motorized Leica AF 6000LX microscope equipped with a ×10 objective. Time-lapse images were acquired every 30 min over a period of 14 h using a cooled CCD (charge-coupled device) camera (Leica Microsystems). The time series was analysed using ImageJ software and cell migration was calculated as the rate of reduction of the free scratched area remaining unoccupied by cells.

N-terminal sequencing of fibulin-5

Fibulin-5-V5-His was purified from HEK-293 cells stably transfected with pCEP4-FBLN5 vector culture medium on Ni-NTA columns. Protein samples were subjected to SDS/PAGE (15 % gel) followed by electroblotting on to Immobilon-P PVDF membranes for 2 h at 15 °C and 400 mA in 0.025 M NaHCO₃ buffer (pH 9.0), containing 20 % (v/v) methanol and 0.1 % SDS. Membranes were washed with methanol. Protein bands were

detected by staining with a 0.1 % aqueous solution of Amido Black 10B. Amino acid sequencing was performed at the Wistar proteomics facility (The Wistar Institute, University of Pennsylvania, PA, U.S.A.).

Boyden chamber migration assay

Migration of MEFs in a Boyden chamber assay was studied as described previously [30], with minor modifications, in the presence or absence of 25 nM uPA or 10 % (v/v) FBS. In brief, *Fbln*^{5+/+}, *Fbln*^{-/-} and *Fbln*^{RGE/RGE} MEFs were preloaded with calcein (Invitrogen) for 30 min, trypsinized, washed and suspended in fresh DMEM containing 0.5 % FBS. Porous membranes (8 µm pore size) were coated with collagen I and MEFs (20 000 cells/50 µl) were placed into the upper chamber. Control (DMEM supplemented with 0.5 % FBS) or recombinant uPA forms (25 nM) were placed into the lower chamber for 3 h. Non-migrating cells were removed and migrating cells were quantified using a 96-well fluorescence plate reader. Chemotactic activity was expressed either as relative fluorescence, reflecting the number of cells, or as the fold stimulation relative to basal migration in the absence of scuPA or FBS.

Cell proliferation assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay was performed according to the manufacturer's protocol. In brief, 5×10³ cells were seeded per well in 96-well plates and cultured in DMEM containing 10 % (v/v) FBS or 0.5 % FBS for 48 h. MTT reagent (10 µl) was added to each well for 4 h at 37 °C under 5 % CO₂. The MTT metabolic product formazan was resuspended in 25 µl of DMSO and measured at 570 nm.

Statistical analysis

Data were analysed by one-way ANOVA with Bonferroni's post-hoc test or Student's *t* test as appropriate using GraphPad Prism 4.01 software (GraphPad Software). Results are means ± S.D. *P* < 0.05 was considered to be statistically significant.

RESULTS

Fibulin-5 is a novel uPA-binding protein

To extend our understanding of the molecular basis of uPA-mediated cell migration during vascular remodelling, we searched for novel uPA-binding partners. As uPAR is a prevalent uPA-binding protein with a high affinity for uPA [4], we addressed this question using a recombinant uPA mutant lacking the uPAR-binding domain GFD (uPA- GFD) to pull-down additional uPA-interacting proteins in SMCs derived from the medial layer of human aortae. uPA- GFD was coupled to Sepharose, aortic lysates were incubated with this affinity matrix and bound proteins were eluted and separated using SDS/PAGE. A protein with an apparent molecular mass of 65 kDa was detected (band ii, Figure 1A) in the eluates from the uPA- GFD-Sepharose, but not from the BSA-Sepharose. The protein was identified by HPLC ESI-MS/MS as human fibulin-5 (Supplementary Table S1 at <http://www.BiochemJ.org/bj/443/bj4430491add.htm>). In addition, we also identified band i

(Figure 1A) as microfibril-associated glycoprotein 4 and band iii (Figure 1A), which corresponded to uPA- GFD (Supplementary Table S1).

To confirm these results, we constructed a vector encoding Myc-tagged fibulin-5 (fibulin-5-c-Myc) and transiently expressed this protein in CHO cells. Figure 1(B) shows that fibulin-5-c-Myc is detected in the cell culture medium and binds to uPA- GFD-Sepharese. To further confirm the binding of uPA to fibulin-5, we analysed their interaction in solution using a pull-down assay with biotinylated uPA. We added biotinylated uPA to the culture medium of CHO cells transfected with pCMV-FBLN5; fibulin-5-c-Myc was immunoprecipitated with an anti-c-Myc antibody. Figure 1(C) shows that biotinylated uPA was pulled down with fibulin-5-c-Myc, but not with a control antibody. The specificity of the fibulin-5-uPA interaction was analysed further using a ligand blotting assay with biotinylated uPA as the detecting probe. Figure 1(D) shows that the biotinylated uPA binds a protein with an apparent molecular mass of 65 kDa, which corresponds to fibulin-5-c-Myc. We then measured the affinity of ^{125}I -labelled uPA binding to recombinant fibulin-5 using a solid-phase assay and found that ^{125}I -labelled uPA bound specifically to fibulin-5 with a K_d of 82 ± 15 nM (Figure 1E). Binding of fibulin-5 to other proteins, such as tropoelastin, requires Ca^{2+} [28]. Therefore we next asked whether the interaction of fibulin-5 with uPA is also Ca^{2+} -dependent. As shown in Figure 1(F), the binding of fibulin-5-c-Myc to immobilized scuPA was abrogated in the presence of EDTA and was restored by the addition of Ca^{2+} , whereas addition of Mg^{2+} had no effect. These results indicate that the high-affinity interaction between uPA and fibulin-5 is Ca^{2+} -dependent.

Mapping the binding domains in uPA and fibulin-5

uPA and fibulin-5 are both multidomain proteins. Therefore we sought to characterize the domains required for their stable interaction. Recombinant uPA variants (Figure 2A) were immobilized on Sepharose and incubated with fibulin-5-c-Myc-containing cell culture media. As shown in Figure 2(B), the C-terminal protease domain of uPA is required for the interaction with fibulin-5.

Fibulin-5 contains six cbEGF domains followed by a C-terminal globular module [40]. We mapped its uPA-binding site using fibulin-5-deletion mutants [41]. Lysates of HEK-293 cells transiently expressing fibulin-5 mutants (Figure 2C) were incubated with recombinant scuPA coupled to Sepharose and the bound proteins were analysed using Western blotting. As shown in Figure 2(D), scuPA precipitated full-length fibulin-5 and all of the mutants with an intact C-terminal domain. Deletion of 48 C-terminal amino acids of fibulin-5 did not affect the interaction, and only the fibulin-5 mutant lacking the entire C-terminal domain was unable to bind uPA.

These studies show that fibulin-5 binds to the single-chain form of uPA, which does not form an active protease catalytic site. We next asked whether fibulin-5 also bound to the catalytically active tcuPA form. Plasmin readily converted immobilized scuPA into tcuPA, as revealed by SDS/PAGE analysis under reducing conditions (Supplementary Figure S1A at <http://www.BiochemJ.org/bj/443/bj4430491add.htm>). In order to confirm that conversion of immobilized scuPA into tcuPA did not result in partial loss of the protein from the affinity matrix, aliquots of the scuPA and tcuPA matrices were incubated with the lysates of

uPAR-expressing HEK-293 cells (HEK-uPAR). Supplementary Figure S1(B) shows that plasmin-activated immobilized tcuPA retains its ability to bind uPAR to the same extent as immobilized scuPA. Importantly, the small protease inhibitor AEBSF [4-(2-aminoethyl)benzenesulfonyl fluoride], which binds and inhibits tcuPA, did not abrogate binding of fibulin-5 (Figure 2E), suggesting that fibulin-5 binds to a site in the proteolytic domain of uPA that is distinct from the serine protease active centre. We confirmed these data using a solid-phase binding assay (Figure 2F).

We also examined whether plasminogen, a substrate of uPA, interferes with binding of fibulin-5. To do this, we incubated uPA immobilized on Sepharose with fibulin-5 in the presence or absence of plasminogen. Aprotinin was added to prevent the conversion of scuPA into tcuPA by plasmin, generated upon co-incubation of scuPA and plasminogen, and degradation of fibulin-5 by plasmin (see below). Our data indicate that plasminogen does not interfere with binding of fibulin-5 to uPA (Figure 2G). Taken together, these results show that fibulin-5 binds to the protease domain of uPA and that the uPA active centre is not involved in this interaction.

Cleavage of fibulin-5

The data described above indicate that uPA bound to fibulin-5 retains the capacity to bind plasminogen and generate plasmin. We next examined whether uPA or other components of the plasminogen activator system can directly cleave fibulin-5. Full-length recombinant V5-tagged human fibulin-5 was purified from the supernatants of HEK-293 cells stably transfected with pCEP4-FBLN5 vector and incubated with tcuPA, tPA or plasmin. The results shown in Supplementary Figure S1(C) demonstrate time-dependent accumulation of the truncated form of fibulin-5 in the presence of plasmin, but not tcuPA or tPA, indicating that fibulin-5 is susceptible to cleavage by plasmin (Supplementary Figure S1C). Since the recombinant fibulin-5 variant we used possesses a C-terminal V5 tag, and both full-length and truncated forms were visualized with an anti-V5 antibody on Western blot, we infer that the cleavage site is located near N-terminus. Our results demonstrate that scuPA binds both full-length and truncated forms of fibulin-5 (Supplementary Figure S1D). In order to confirm that plasmin is responsible for the fibulin-5 cleavage, we performed an analysis of inhibition using a panel of related protease inhibitors. HEK-293 cells were transfected with the pCEP-FBLN5 vector encoding human V5-tagged fibulin-5, and incubated for 48 h. We observed both intact and truncated forms of fibulin-5 in the conditioned medium of transfected cells (Supplementary Figure S1E). Fibulin-5 cleavage was almost completely blocked in the presence of aprotinin (which inhibits plasmin, kallikrein and some other serine proteases, but not uPA) and α_2 -antiplasmin (a specific plasmin inhibitor), whereas PAI-1 (plasminogen-activator inhibitor-1) only partially inhibited processing of fibulin-5, and amiloride, a specific inhibitor of uPA, had no effect (Supplementary Figure S1E). Truncated fibulin-5 also interacted with the rabbit polyclonal anti-fibulin-5 antibody BSYN [28], which was generated against a peptide located within the linker region between the first and second cbEGF domains in rat fibulin-5 (Supplementary Figure S2A at <http://www.BiochemJ.org/bj/443/bj4430491add.htm>), suggesting that plasmin cleaves fibulin-5 within its first N-terminal cbEGF domain.

Accumulation of a truncated form of fibulin-5 lacking the integrin-binding domain formed by cleavage after Arg⁷⁷ has been reported previously [33]. To confirm that plasmin cleavage removes the first RGD-containing cbEGF domain [33], we purified both full-length and truncated V5-tagged fibulin-5 proteins. The two species were separated by SDS/PAGE, transferred on to a PVDF membrane and visualized with Amido Black dye or anti-V5 antibody (Supplementary Figure S1F). The band containing the truncated form was excised and subjected to partial N-terminal amino acid sequencing. Two predominant N-terminal sequences were identified, S⁸⁹GPYPAAA and S⁸⁵TPYSGPY, which are located within the linker region between the first and second cbEGF domains in fibulin-5, and the cleavage sites Y⁸⁵S and Y⁸⁹S, consistent with the proteolytic specificity of chymotrypsin-like serine protease (Supplementary Figure S1F).

Fibulin-5 co-localizes with uPA on the cell surface and mediates uPA-induced cell migration

uPA and fibulin-5 co-localized on the surface of PSMCs, as assessed by dual immunofluorescence labelling (Figure 3A). uPA promotes cell adhesion and migration via proteolytic and cell signalling mechanisms [3,22,30]. Therefore we asked whether fibulin-5 mediates uPA-dependent migration using MEFs isolated from the wild-type (*Fbln*^{+/+}) and fibulin-5-knockout (*Fbln*^{-/-}) mice [28]. Mouse uPA (25 nM) increased by 1.5-fold the transmigration of MEFs from the *Fbln*^{+/+} mice through collagen I-coated membranes in the 8 μ m pore size Transwell membrane system, whereas it did not affect transmigration of MEFs from *Fbln*^{-/-} mice (Figure 3B and Supplementary Figure S2A).

To explore the effect of fibulin-5 on uPA-stimulated cell migration under conditions that simulate wound healing, we performed a scratch assay in the presence or absence of 25 nM uPA. MEFs easily broke cell-cell contacts and migrated into the scratched cell-free region. As shown in Figure 3(C), uPA stimulated migration of MEFs from *Fbln*^{+/+} mice, but did not affect migration of *Fbln*^{-/-} MEFs. This difference could not be explained by differences in the cell numbers, because the proliferation rates of both cells types were equal (Supplementary Figure S2B). As would be expected from the inverse relationship between cell adhesion and migration [42], the faster migration of wild-type MEFs in the scratch assay was consistent with their lower adhesivity compared with fibulin-5-deficient cells (Supplementary Figure S3A at <http://www.BiochemJ.org/bj/443/bj4430491add.htm>).

uPAR is an important mediator of uPA-dependent cell migration [43]. Binding of fibulin-5 to the proteolytic domain of uPA suggests that simultaneous interaction of uPA with uPAR through its GFD and with fibulin-5 through its catalytic domain could be crucial for the formation of a functional signalling protein complex, which initiates uPA-dependent migration. To obtain greater insight into the mechanisms of fibulin-5-mediated migration of MEFs stimulated by uPA, we examined the role of uPAR in fibulin-5-dependent migration. It is known that human uPA does not bind to mouse uPAR. Therefore we compared migration of *Fbln*^{+/+} and *Fbln*^{-/-} MEFs in response to murine and human uPA. Both uPA forms stimulated migration of *Fbln*^{+/+} MEFs in the Boyden chamber assay, indicating that uPAR is not essential for this process (Figure 3D), whereas neither human nor mouse uPA

stimulated migration of *Fbln5*^{-/-} MEFs, and serum stimulated the migration of both cell lines to the same extent (Figure 3D).

We also observed that basal migration of non-stimulated *Fbln5*^{-/-} MEFs was markedly higher ($P < 0.001$) than that of the *Fbln5*^{+/+} MEFs (Figure 3B). Addition of purified fibulin-5 protein to *Fbln5*^{-/-} MEFs significantly decreased basal migration, reflected by a lower number of transmigrated cells (Figure 4A). Addition of 25 nM uPA to *Fbln5*^{-/-} MEFs in the presence of exogenously added fibulin-5 restored the motility of these cells to the extent exhibited by intact *Fbln5*^{-/-} MEFs (Figures 4A and 4B). This difference in the basal migration of MEFs from *Fbln5*^{-/-} and *Fbln5*^{+/+} prompted us to investigate whether fibulin-5 inhibits migration by dysregulating the function(s) of selected integrins, which maintain cell motility. Therefore we compared the migration of MEFs isolated from mice in which the integrin-binding RGD motif in fibulin-5 was mutated (*Fbln5*^{RGE/RGE}) [44] with their wild-type littermates (*Fbln5*^{wt/wt}). Basal migration of *Fbln5*^{RGE/RGE} MEFs was higher than that of the *Fbln5*^{wt/wt} MEFs; however, uPA did not stimulate the migration of *Fbln5*^{RGE/RGE} MEFs (Figures 4C and 4D), similar to the lack of response by *Fbln5*^{-/-} MEFs (Figure 3B). Thus the binding of fibulin-5 to integrins affects both cell motility and their capability to migrate in response to uPA.

Fibulin-5 binds β 1-integrins (α 5 β 1 and α 4 β 1), but does not support their activation in SMCs [45], in contrast with their natural ligand fibronectin. The ability of uPA to stimulate cell migration involves a number of pathways which includes extracellular proteolysis, plasmin generation and activation of downstream intracellular signalling which, in turn, might be mediated by integrins [22,30,46]. Thus it is possible that fibulin-5 mediates uPA-stimulated cell migration by modulation of integrin function. We found that blocking of β 1-integrin with an anti-(human β 1-integrin) antibody inhibited both basal motility and migration of PSMCs in response to uPA (Figure 4E). To test whether plasmin generation is involved in fibulin-5-mediated cell motility, we used a catalytically inactive variant of uPA, (S356A)scuPA, which is unable to activate plasminogen. We found that (S356A)scuPA induced the migration of PSMCs to a much lower extent than wild-type scuPA (Figure 4E). Figures 4(F) and 4(G) show that aprotinin, an inhibitor of plasmin and other serine proteases, also reduces the motility of wild-type MEFs in response to uPA, suggesting that the catalytic activity of plasmin at least partially mediates the pro-migratory effect of uPA. Taken together these results show that fibulin-5-mediated migration of cells in response to uPA depends on β 1-integrins and requires plasmin generation.

In order to elucidate whether incubation of cells with uPA causes cleavage and dissociation of fibulin-5 from β 1-integrins, HEK-293 cells transfected with the pCEP4-FBLN5 vector, which overexpress fibulin-5-V5 protein, were incubated with uPA for 4 h, and β 1-integrin was immunoprecipitated from the cell lysates. Figure 5(A) shows that the β 1-integrin subunit co-immunoprecipitated with fibulin-5 in untreated cells, but was not associated with fibulin-5 in uPA-treated cells. Incubation of these cells with scuPA for 4 h led to accumulation of the truncated form of fibulin-5 (Figure 5B), which does not bind integrins due to elimination of the RGD-containing N-terminal cbEGF domain. We also observed that endogenous fibulin-5 and the β 1-integrin subunit partially co-localize in untreated PSMCs, but not in PSMCs treated with uPA for 4 h (Figure 5C). Furthermore, overexpression of

the non-cleavable mutant of fibulin-5 [33] decreased the ability of uPA to induce PSMC migration (Figure 4E and Supplementary Figure S4 at <http://www.BiochemJ.org/bj/443/bj4430491add.htm>). Taken together, these results suggest that uPA-mediated cleavage of fibulin-5 by plasmin unblocks β 1-integrin signalling, which facilitates cell motility.

DISCUSSION

We have identified fibulin-5 as a novel uPA-binding protein involved in the regulation of cell motility downstream of uPA. Fibulin-5 is an extracellular matrix integrin-binding protein which regulates formation of elastic fibres and modulates cell adhesion and migration [28,47,48]. Using various deletion mutants of uPA, we determined that fibulin-5 binds to the proteolytic domain of uPA. However, we did not observe proteolytic cleavage of fibulin-5 by uPA, suggesting that fibulin-5 is not an uPA substrate. We also demonstrate that plasminogen, the major substrate of uPA, does not compete with fibulin-5 for binding to uPA. Moreover, the small protease inhibitor AEBSF did not prevent binding of scuPA and tcuPA to fibulin-5.

However, incubation of cells with uPA facilitated partial proteolytic processing of fibulin-5. Using a set of protease inhibitors, we found that plasmin, which is converted from plasminogen by uPA, mediates fibulin-5 cleavage which eliminates the first cbEGF domain. An *in vivo* accumulation of a truncated form of fibulin-5 lacking the first N-terminal cbEGF domain cleaved between Arg⁷⁷ and Gly⁷⁸ by a serine protease-like enzyme has been reported [33]. Moreover, elimination of the N-terminal cbEGF domain abrogates the elastogenic properties of fibulin-5 [33]. Interestingly, we identified two N-terminal sequences in the linker region between the first and second cbEGF domains in fibulin-5, S⁸⁹GPYPAAA and S⁸⁵TPYSGPY, which correspond to the original cleavage sites Y⁸⁵S and Y⁸⁹S respectively. Although the site identified does not represent a typical plasmin-cleavage site, we suggest that plasmin, as a broad-substrate-specific protease, could simultaneously cleave fibulin-5 at Arg⁷⁷ as reported previously [33] and activate other chymotrypsin-like proteases in serum or on the surface of HEK-293 cells to generate these additional truncated forms. Alternatively, the initial cleavage of fibulin-5 at Arg⁷⁷ may permit subsequent partial proteolytic processing by chymotrypsin-like proteases. Thus uPA binding to fibulin-5 initiates a proteolytic cascade which results in elimination of an integrin-binding RGD-containing cbEGF domain first from fibulin-5.

Fibulin-5 is distinguished from other fibulins by the presence of an evolutionarily conserved integrin-binding RGD motif within its first cbEGF domain, which mediates binding to a subset of integrins, including α 5 β 1, α v β 3, α 9 β 1 and α v β 5 [45, 47]. Fibulin-5 also enhances substrate attachment of endothelial cells. Fibulin-5 mediates attachment and spreading of primary aortic SMCs through binding to the fibronectin receptor α 5 β 1 and α 4 β 1, but not to α v β 3 [45]. However, fibulin-5 fails to activate downstream signalling after binding to α 5 β 1 and α 4 β 1 integrins, suggesting that fibulin-5 may act in a dominant-negative fashion to inhibit fibronectin receptor-mediated signalling. On the other hand, loss of fibulin-5 binding to β 1-integrins prevents pancreatic tumour growth by increasing the levels of ROS (reactive oxygen species) [49]. These results suggest that readouts of the cross-talk between fibulin-5 and different subsets of integrins are cell-type-and context-specific.

In the present study we show that the uPA–fibulin-5 interaction, uPA-mediated plasminogen activation and partial proteolytic processing of fibulin-5 releasing the integrin-binding domain act in concert to promote cell migration. uPA regulates cell migration via multiple mechanisms, including binding to uPAR and initiating intracellular signalling cascades [50,51], via urokinase kringle-dependent mechanisms [30,46,52] and proteolytic mechanisms [22]. Signal transduction initiated by the uPA–uPAR complex depends on multiple interactions of uPAR with co-receptors and integrins [3,23,24,53]. Binding of uPA to uPAR results in its redistribution to the leading edge of migrating cells [54] and enhances its association with integrins, which in turn augments integrin-mediated signalling and cell motility [55–58]. Both uPA [46] and fibulin-5 [59] regulate cell migration and activate integrin-dependent signalling pathways. The results of the present study strongly support the hypothesis that fibulin-5 mediates uPA-induced cell migration via the modulation of integrin-dependent signalling pathways in an uPAR-independent manner. First, we used *Fbln5*^{+/+} and *Fbln5*^{-/-} MEFs to demonstrate that cell migration in response to uPA is fibulin-5-dependent (Figure 3B). Our results demonstrate that *Fbln5*^{+/+} MEFs were able to migrate in response to both human and mouse uPA. Assuming that human uPA does not bind mouse uPAR, the results indicate that binding of uPA to uPAR is not essential for uPA-dependent migration of MEFs. Moreover, our experimental setting for examining migration involved initial detachment with trypsin/EDTA to prepare cell suspensions, which is known to eliminate uPAR from the cell surface [60]. This helps to explain the contribution of an uPAR-independent mechanism in cell migration in the experimental setting used in the present study, although further studies are needed to determine whether the uPA interaction with fibulin-5 has any effects on uPAR–integrin-dependent signalling pathways and cell migration *in vivo*. Secondly, we observed that uPA did not stimulate transmigration of *Fbln5*^{RGE/RGE} MEFs in contrast with *Fbln5*^{wt/wt} MEFs. However, we observed that both *Fbln5*^{-/-} MEFs and *Fbln5*^{RGE/RGE} MEFs exhibit higher basal motility than their wild-type MEF counterparts. Higher motility of *Fbln5*^{-/-} vascular SMCs has been reported previously [48]. Given that fibulin-5 fails to activate downstream signalling upon binding to $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins [45] and inhibits the $\beta 1$ -integrin-dependent fibronectin-mediated up-regulation of MMP (matrix metalloproteinase)-9 in murine vaginal stromal cells [44], we hypothesize that fibulin-5 acts in a dominant-negative fashion to uncouple fibronectin– $\beta 1$ -integrin-mediated adhesion/motility signalling. In this case, the higher basal motility of *Fbln5*^{-/-} MEFs and *Fbln5*^{RGE/RGE} MEFs might be attributed to the reversal of the signalling capacity of the fibronectin– $\beta 1$ -integrin complex to maintain cell motility.

Addition of fibulin-5 to *Fbln5*^{-/-} MEFs inhibited basal transmigration, whereas uPA restored the motility of fibulin-5-treated *Fbln5*^{-/-} MEFs to the same extent as intact *Fbln5*^{-/-} MEFs. We suggest that uPA induces the migration of fibulin-5-expressing cells by activating a cascade of local proteolysis, which results in cleavage of uPA-bound fibulin-5 as described above, dissociation of the latter from the $\beta 1$ -integrins and re-coupling of fibronectin– $\beta 1$ -integrin signalling, leading to enhanced cell motility. Indeed, addition of a blocking anti-($\beta 1$ -integrin) antibody significantly inhibited basal migration and abrogated uPA-dependent migration of human PSMCs in response to uPA. This hypothesis is further supported by the fact that the $\beta 1$ -integrin subunit co-immunoprecipitates and co-localizes with fibulin-5 in untreated cells, but not in uPA-treated cells. Moreover, the results of the present study

demonstrate that proteolytic activity is one of the components required for PASMC migration in response to uPA, as the catalytically inactive mutant (S365A)scuPA possesses significantly lower chemotactic capacity than wild-type uPA, and overexpression of the non-cleavable mutant of fibulin-5 in PASMCs reduced migration in response to uPA.

Binding of uPA to fibulin-5 described in the present paper might be implicated in a number of pathophysiological processes, including vasculoproliferative disorders such as restenosis, angiogenesis and pulmonary artery hypertension. Overexpression of uPA shortly after intravascular injury is linked to increased proliferation and migration of the activated SMCs resulting in neointima formation [21]. Overexpression of fibulin-5 in response to injury might represent a compensatory protective mechanism [40]. However, this regulatory effect might be overridden by uPA-mediated cleavage of fibulin-5, which unmasks β 1-integrin signalling, increasing SMC motility, which facilitates vascular remodelling. Results from experimental models of pulmonary hypertension in *Plau*^{-/-} and *Plg*^{-/-} mice indicate that up-regulation of uPA is critical for pulmonary vascular remodelling [61,62]. Our results suggest that proteolytic processing of fibulin-5 and its regulation of β 1-integrin activation may be important intermediaries in this process [44]. Finally, the antagonistic function of fibulin-5 in angiogenesis has been demonstrated *in vitro* [63] and *in vivo* [64]; fibulin-5 may block angiogenesis by inducing the anti-angiogenic molecule thrombospondin-1, by antagonizing VEGF-165-mediated signalling, and/or by antagonizing fibronectin-mediated signalling through direct binding and blocking of the α 5 β 1 fibronectin receptor. uPA binding and cleavage of fibulin-5, which releases its integrin-binding N-terminal cbEGF domain, could antagonize the anti-angiogenic capacity of fibulin-5 and also contribute to the pro-angiogenic activity of uPA [65].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
CHO	Chinese-hamster ovary
DMEM	Dulbecco's modified Eagle's medium

DPBS	Dulbecco's PBS
ESI	electrospray ionization
FBS	fetal bovine serum
GFD	growth factor-like domain
GFP	green fluorescent protein
HEK	human embryonic kidney
HRP	horseradish peroxidase
MEF	mouse embryonic fibroblast
MS/MS	tandem MS
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 <i>H</i> -tetrazolium bromide
Ni-NTA	Ni ²⁺ -nitrilotriacetate
PASMC	pulmonary arterial smooth muscle cell
SMC	smooth muscle cell
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
scuPA	single-chain uPA
tcuPA	two-chain uPA
uPAR	uPA receptor
VEGF	vascular endothelial growth factor

References

1. Tkachuk VA, Plekhanova OS, Parfyonova YV. Regulation of arterial remodeling and angiogenesis by urokinase-type plasminogen activator. *Can J Physiol Pharmacol.* 2009; 87:231–251. [PubMed: 19370078]
2. Smith HW, Marshall CJ. Regulation of cell signalling by uPAR. *Nat Rev Mol Cell Biol.* 2010; 11:23–36. [PubMed: 20027185]
3. Blasi F, Sidenius N. The urokinase receptor: focused cell surface proteolysis, cell adhesion and signaling. *FEBS Lett.* 2010; 584:1923–1930. [PubMed: 20036661]
4. Appella E, Robinson EA, Ullrich SJ, Stoppelli MP, Corti A, Cassani G, Blasi F. The receptor-binding sequence of urokinase. A biological function for the growth-factor module of proteases. *J Biol Chem.* 1987; 262:4437–4440. [PubMed: 3031025]
5. Pannell R, Gurewich V. Activation of plasminogen by single-chain urokinase or by two-chain urokinase: a demonstration that single-chain urokinase has a low catalytic activity (pro-urokinase). *Blood.* 1987; 69:22–26. [PubMed: 3790724]
6. Husain SS. Single-chain urokinase-type plasminogen activator does not possess measurable intrinsic amidolytic or plasminogen activator activities. *Biochemistry.* 1991; 30:5797–5805. [PubMed: 1828371]
7. Kasai S, Arimura H, Nishida M, Suyama T. Proteolytic cleavage of single-chain pro-urokinase induces conformational change which follows activation of the zymogen and reduction of its high affinity for fibrin. *J Biol Chem.* 1985; 260:12377–12381. [PubMed: 3930494]

8. Naldini L, Vigna E, Bardelli A, Follenzi A, Galimi F, Comoglio PM. Biological activation of pro-HGF (hepatocyte growth factor) by urokinase is controlled by a stoichiometric reaction. *J Biol Chem.* 1995; 270:603–611. [PubMed: 7822285]
9. Plouet J, Moro F, Bertagnolli S, Coldeboeuf N, Mazarguil H, Clamens S, Bayard F. Extracellular cleavage of the vascular endothelial growth factor 189-amino acid form by urokinase is required for its mitogenic effect. *J Biol Chem.* 1997; 272:13390–13396. [PubMed: 9148962]
10. Vassalli JD, Sappino AP, Belin D. The plasminogen activator/plasmin system. *J Clin Invest.* 1991; 88:1067–1072. [PubMed: 1833420]
11. Saksela O, Rifkin DB. Release of basic fibroblast growth factor-heparan sulfate complexes from endothelial cells by plasminogen activator-mediated proteolytic activity. *J Cell Biol.* 1990; 110:767–775. [PubMed: 2137829]
12. Chapman HA. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. *Curr Opin Cell Biol.* 1997; 9:714–724. [PubMed: 9330876]
13. Bdeir K, Murciano JC, Tomaszewski J, Koniaris L, Martinez J, Cines DB, Muzykantov VR, Higazi AA. Urokinase mediates fibrinolysis in the pulmonary microvasculature. *Blood.* 2000; 96:1820–1826. [PubMed: 10961882]
14. Yamamoto K, Loskutoff DJ. Fibrin deposition in tissues from endotoxin-treated mice correlates with decreases in the expression of urokinase-type but not tissue-type plasminogen activator. *J Clin Invest.* 1996; 97:2440–2451. [PubMed: 8647936]
15. Carmeliet P, Moons L, Ploplis V, Plow E, Collen D. Impaired arterial neointima formation in mice with disruption of the plasminogen gene. *J Clin Invest.* 1997; 99:200–208. [PubMed: 9005988]
16. Gutierrez LS, Schulman A, Brito-Robinson T, Noria F, Ploplis VA, Castellino FJ. Tumor development is retarded in mice lacking the gene for urokinase-type plasminogen activator or its inhibitor, plasminogen activator inhibitor-1. *Cancer Res.* 2000; 60:5839–5847. [PubMed: 11059781]
17. Ploplis VA, Castellino FJ. Gene targeting of components of the fibrinolytic system. *Thromb Haemostasis.* 2002; 87:22–31. [PubMed: 11848453]
18. Lupu F, Heim DA, Bachmann F, Hurni M, Kakkar VV, Kruithof EK. Plasminogen activator expression in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol.* 1995; 15:1444–1455. [PubMed: 7670960]
19. Kienast J, Padro T, Steins M, Li CX, Schmid KW, Hammel D, Scheld HH, van de Loo JC. Relation of urokinase-type plasminogen activator expression to presence and severity of atherosclerotic lesions in human coronary arteries. *Thromb Haemostasis.* 1998; 79:579–586. [PubMed: 9531045]
20. Padro T, Emeis JJ, Steins M, Schmid KW, Kienast J. Quantification of plasminogen activators and their inhibitors in the aortic vessel wall in relation to the presence and severity of atherosclerotic disease. *Arterioscler Thromb Vasc Biol.* 1995; 15:893–902. [PubMed: 7600121]
21. Plekhanova O, Parfyonova Y, Bibilashvily R, Domogatskii S, Stepanova V, Gulba DC, Agrotis A, Bobik A, Tkachuk V. Urokinase plasminogen activator augments cell proliferation and neointima formation in injured arteries via proteolytic mechanisms. *Atherosclerosis.* 2001; 159:297–306. [PubMed: 11730809]
22. Stepanova V, Mukhina S, Kohler E, Resink TJ, Erne P, Tkachuk VA. Urokinase plasminogen activator induces human smooth muscle cell migration and proliferation via distinct receptor-dependent and proteolysis-dependent mechanisms. *Mol Cell Biochem.* 1999; 195:199–206. [PubMed: 10395084]
23. Okada SS, Grobmyer SR, Barnathan ES. Contrasting effects of plasminogen activators, urokinase receptor, and LDL receptor-related protein on smooth muscle cell migration and invasion. *Arterioscler Thromb Vasc Biol.* 1996; 16:1269–1276. [PubMed: 8857924]
24. Tarui T, Akakura N, Majumdar M, Andronicos N, Takagi J, Mazar AP, Bdeir K, Kuo A, Yarovoi SV, Cines DB, Takada Y. Direct interaction of the kringle domain of urokinase-type plasminogen activator (uPA) and integrin $\alpha v \beta 3$ induces signal transduction and enhances plasminogen activation. *Thromb Haemostasis.* 2006; 95:524–534. [PubMed: 16525582]

25. Carmeliet P, Moons L, Herbert JM, Crawley J, Lupu F, Lijnen R, Collen D. Urokinase but not tissue plasminogen activator mediates arterial neointima formation in mice. *Circ Res.* 1997; 81:829–839. [PubMed: 9351457]
26. Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, Mulligan RC. Physiological consequences of loss of plasminogen activator gene function in mice. *Nature.* 1994; 368:419–424. [PubMed: 8133887]
27. Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, Dyspersin GD, Cleutjens JP, Shipley M, Angellilo A, et al. Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. *Nat Med.* 1999; 5:1135–1142. [PubMed: 10502816]
28. Yanagisawa H, Davis EC, Starcher BC, Ouchi T, Yanagisawa M, Richardson JA, Olson EN. Fibulin-5 is an elastin-binding protein essential for elastic fibre development *in vivo*. *Nature.* 2002; 415:168–171. [PubMed: 11805834]
29. Todaro GJ, Green H. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J Cell Biol.* 1963; 17:299–313. [PubMed: 13985244]
30. Mukhina S, Stepanova V, Traktouev D, Poliakov A, Beabealashvili R, Gursky Y, Minashkin M, Shevelev A, Tkachuk V. The chemotactic action of urokinase on smooth muscle cells is dependent on its kringle domain. Characterization of interactions and contribution to chemotaxis. *J Biol Chem.* 2000; 275:16450–16458. [PubMed: 10749881]
31. Poliakov A, Tkachuk V, Ovchinnikova T, Potapenko N, Bagryantsev S, Stepanova V. Plasmin-dependent elimination of the growth-factor-like domain in urokinase causes its rapid cellular uptake and degradation. *Biochem J.* 2001; 355:639–645. [PubMed: 11311125]
32. Wang XQ, Bdeir K, Yarovoi S, Cines DB, Fang W, Abraham E. Involvement of the urokinase kringle domain in lipopolysaccharide-induced acute lung injury. *J Immunol.* 2006; 177:5550–5557. [PubMed: 17015742]
33. Hirai M, Ohbayashi T, Horiguchi M, Okawa K, Hagiwara A, Chien KR, Kita T, Nakamura T. Fibulin-5/DANCE has an elastogenic organizer activity that is abrogated by proteolytic cleavage *in vivo*. *J Cell Biol.* 2007; 176:1061–1071. [PubMed: 17371835]
34. Stepanova V, Lebedeva T, Kuo A, Yarovoi S, Tkachuk S, Zaitsev S, Bdeir K, Dumler I, Marks MS, Parfyonova Y, et al. Nuclear translocation of urokinase-type plasminogen activator. *Blood.* 2008; 112:100–110. [PubMed: 18337556]
35. Kuzmenko YS, Bochkov VN, Philippova MP, Tkachuk VA, Resink TJ. Characterization of an atypical lipoprotein-binding protein in human aortic media membranes by ligand blotting. *Biochem J.* 1994; 303:281–287. [PubMed: 7945254]
36. Wait R, Miller I, Eberini I, Cairoli F, Veronesi C, Battocchio M, Gemeiner M, Gianazza E. Strategies for proteomics with incompletely characterized genomes: the proteome of *Bos taurus* serum. *Electrophoresis.* 2002; 23:3418–3427. [PubMed: 12373772]
37. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal Chem.* 2002; 74:5383–5392. [PubMed: 12403597]
38. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal Chem.* 2003; 75:4646–4658. [PubMed: 14632076]
39. Bdeir K, Kuo A, Sachais BS, Rux AH, Bdeir Y, Mazar A, Higazi AA, Cines DB. The kringle stabilizes urokinase binding to the urokinase receptor. *Blood.* 2003; 102:3600–3608. [PubMed: 12881310]
40. Kowal RC, Richardson JA, Miano JM, Olson EN. EVEC, a novel epidermal growth factor-like repeat-containing protein upregulated in embryonic and diseased adult vasculature. *Circ Res.* 1999; 84:1166–1176. [PubMed: 10347091]
41. Zheng Q, Davis EC, Richardson JA, Starcher BC, Li T, Gerard RD, Yanagisawa H. Molecular analysis of fibulin-5 function during *de novo* synthesis of elastic fibers. *Mol Cell Biol.* 2007; 27:1083–1095. [PubMed: 17130242]
42. DiMilla PA, Barbee K, Lauffenburger DA. Mathematical model for the effects of adhesion and mechanics on cell migration speed. *Biophys J.* 1991; 60:15–37. [PubMed: 1883934]

43. Stepanova V, Bobik A, Bibilashvily R, Belogurov A, Rybalkin I, Domogatsky S, Little PJ, Goncharova E, Tkachuk V. Urokinase plasminogen activator induces smooth muscle cell migration: key role of growth factor-like domain. *FEBS Lett.* 1997; 414:471–474. [PubMed: 9315743]
44. Budatha M, Roshanravan S, Zheng Q, Weislander C, Chapman SL, Davis EC, Starcher B, Word RA, Yanagisawa H. Extracellular matrix proteases contribute to progression of pelvic organ prolapse in mice and humans. *J Clin Invest.* 2011; 121:2048–2059. [PubMed: 21519142]
45. Lomas AC, Mellody KT, Freeman LJ, Bax DV, Shuttleworth CA, Kielty CM. Fibulin-5 binds human smooth-muscle cells through $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins, but does not support receptor activation. *Biochem J.* 2007; 405:417–428. [PubMed: 17472576]
46. Goncharova EA, Vorotnikov AV, Gracheva EO, Wang CL, Panettieri RA Jr, Stepanova VV, Tkachuk VA. Activation of p38 MAP-kinase and caldesmon phosphorylation are essential for urokinase-induced human smooth muscle cell migration. *Biol Chem.* 2002; 383:115–126. [PubMed: 11930938]
47. Nakamura T, Lozano PR, Ikeda Y, Iwanaga Y, Hinek A, Minamisawa S, Cheng CF, Kobuke K, Dalton N, Takada Y, et al. Fibulin-5/DANCE is essential for elastogenesis *in vivo*. *Nature.* 2002; 415:171–175. [PubMed: 11805835]
48. Spencer JA, Hacker SL, Davis EC, Mecham RP, Knutsen RH, Li DY, Gerard RD, Richardson JA, Olson EN, Yanagisawa H. Altered vascular remodeling in fibulin-5-deficient mice reveals a role of fibulin-5 in smooth muscle cell proliferation and migration. *Proc Natl Acad Sci USA.* 2005; 102:2946–2951. [PubMed: 15710889]
49. Schluterman MK, Chapman SL, Korpanty G, Ozumi K, Fukai T, Yanagisawa H, Brekken RA. Loss of fibulin-5 binding to $\beta 1$ integrins inhibits tumor growth by increasing the level of ROS. *Dis Model Mech.* 2010; 3:333–342. [PubMed: 20197418]
50. Kusch A, Tkachuk S, Haller H, Dietz R, Gulba DC, Lipp M, Dumler I. Urokinase stimulates human vascular smooth muscle cell migration via a phosphatidylinositol 3-kinase-Tyk2 interaction. *J Biol Chem.* 2000; 275:39466–39473. [PubMed: 10995743]
51. Dumler I, Weis A, Mayboroda OA, Maasch C, Jerke U, Haller H, Gulba DC. The Jak/Stat pathway and urokinase receptor signaling in human aortic vascular smooth muscle cells. *J Biol Chem.* 1998; 273:315–321. [PubMed: 9417082]
52. Tanski WJ, Fegley AJ, Roztocil E, Davies MG. Domain-dependent action of urokinase on smooth muscle cell responses. *J Vasc Surg.* 2004; 39:214–222. [PubMed: 14718842]
53. Nassar T, Yarovoi S, Fanne RA, Akkawi S, Jammal M, Allen TC, Idell S, Cines DB, Higazi AA. Regulation of airway contractility by plasminogen activators through *N*-methyl-D-aspartate receptor-1. *Am J Respir Cell Mol Biol.* 2010; 43:703–711. [PubMed: 20097831]
54. Estreicher A, Muhlhauser J, Carpentier JL, Orci L, Vassalli JD. The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. *J Cell Biol.* 1990; 111:783–792. [PubMed: 2166055]
55. Yebra M, Goretzki L, Pfeifer M, Mueller BM. Urokinase-type plasminogen activator binding to its receptor stimulates tumor cell migration by enhancing integrin-mediated signal transduction. *Exp Cell Res.* 1999; 250:231–240. [PubMed: 10388537]
56. Degryse B, Resnati M, Rabbani SA, Villa A, Fazioli F, Blasi F. Src-dependence and pertussis-toxin sensitivity of urokinase receptor-dependent chemotaxis and cytoskeleton reorganization in rat smooth muscle cells. *Blood.* 1999; 94:649–662. [PubMed: 10397732]
57. Mazzei R, D'Alessio S, Kenmoe RK, Ossowski L, Blasi F. An uncleavable uPAR mutant allows dissection of signaling pathways in uPA-dependent cell migration. *Mol Biol Cell.* 2006; 17:367–378. [PubMed: 16267271]
58. Tang CH, Hill ML, Brumwell AN, Chapman HA, Wei Y. Signaling through urokinase and urokinase receptor in lung cancer cells requires interactions with $\beta 1$ integrins. *J Cell Sci.* 2008; 121:3747–3756. [PubMed: 18940913]
59. Schiemann WP, Blobel GC, Kalume DE, Pandey A, Lodish HF. Context-specific effects of fibulin-5 (DANCE/EVEC) on cell proliferation, motility, and invasion. Fibulin-5 is induced by

- transforming growth factor- β and affects protein kinase cascades. *J Biol Chem.* 2002; 277:27367–27377. [PubMed: 12021267]
60. Solberg H, Lober D, Eriksen J, Ploug M, Ronne E, Behrendt N, Dano K, Hoyer-Hansen G. Identification and characterization of the murine cell surface receptor for the urokinase-type plasminogen activator. *Eur J Biochem.* 1992; 205:451–458. [PubMed: 1374026]
61. Levi M, Moons L, Bouche A, Shapiro SD, Collen D, Carmeliet P. Deficiency of urokinase-type plasminogen activator-mediated plasmin generation impairs vascular remodeling during hypoxia-induced pulmonary hypertension in mice. *Circulation.* 2001; 103:2014–2020. [PubMed: 11306532]
62. Heymans S, Lupu F, Terclavers S, Vanwetswinkel B, Herbert JM, Baker A, Collen D, Carmeliet P, Moons L. Loss or inhibition of uPA or MMP-9 attenuates LV remodeling and dysfunction after acute pressure overload in mice. *Am J Pathol.* 2005; 166:15–25. [PubMed: 15631996]
63. Albig AR, Schiemann WP. Fibulin-5 antagonizes vascular endothelial growth factor (VEGF) signaling and angiogenic sprouting by endothelial cells. *DNA Cell Biol.* 2004; 23:367–379. [PubMed: 15231070]
64. Albig AR, Neil JR, Schiemann WP. Fibulins 3 and 5 antagonize tumor angiogenesis *in vivo*. *Cancer Res.* 2006; 66:2621–2629. [PubMed: 16510581]
65. Koolwijk P, van Erck MG, de Vree WJ, Vermeer MA, Weich HA, Hanemaaijer R, van Hinsbergh VW. Cooperative effect of $\text{TNF}\alpha$, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. *J Cell Biol.* 1996; 132:1177–1188. [PubMed: 8601593]

Figure 1. Fibulin-5 is a novel uPA-binding protein

(A) Binding of aortic fibulin-5 to immobilized uPA. Lysates prepared from membranes of human aortae were incubated with a control matrix containing immobilized BSA (lane 1) or with matrix with immobilized uPA- GFD (lane 2). Bound proteins were eluted and separated by SDS/PAGE. The gel was stained with Coomassie Brilliant Blue and protein bands (i–iii, marked by arrows) were excised and subjected to HPLC ESI-MS/MS analysis. (B–D) Fibulin-5–c-Myc expressed in CHO cells binds uPA. (B) Cell culture medium collected from pCMV-FBLN5- or pCMV-GFP-transfected cells was incubated with BSA– (1) or uPA–Sepharose (2). Bound proteins were eluted and analysed by Western blotting using an anti-c-Myc antibody. (C) Biotinylated uPA (6 nM) was added to the cell culture medium of transfected CHO cells containing fibulin-5–c-Myc and immunoprecipitated with an anti-c-Myc antibody immobilized on agarose and analysed by Western blotting. Biotinylated uPA was visualized using HRP-conjugated neutravidin, and fibulin-5–c-Myc fusion protein was visualized with an anti-c-Myc antibody. IgG, unspecific IgG bound to agarose. (D) Cell culture medium from pCMV-FBLN5- or pCMV-GFP-transfected CHO cells were applied on to scuPA–Sepharose and bound proteins were analysed by Western blotting using biotinylated uPA or an anti-c-Myc antibody. (E) Binding of uPA to immobilized human fibulin-5. Recombinant fibulin-5 (5 μ g/ml) or BSA (5 μ g/ml) was immobilized on strip-well plates. 125 I-Labelled uPA- GFD (0.5–400 nM) was added alone or in the presence of a 100-fold molar excess of unlabelled uPA- GFD for 1 h. Wells were washed with PBS and the bound radioactivity was determined. Values are the means $\pm$ S.D.

from two independent experiments performed in triplicate. **(F)** The uPA–fibulin-5 interaction is Ca^{2+} -dependent. Aliquots of cell culture medium containing fibulin-5–c-Myc secreted by transfected CHO cells were incubated with scuPA–Sephadex in the absence or presence of 2 mM EDTA, 5 mM CaCl_2 or 5 mM MgCl_2 . Bound proteins were eluted in sample buffer, subjected to Western blotting and probed with an anti-c-Myc antibody. For the Western blots, the molecular mass in kDa is indicated on the left-hand side.

Figure 2. Domain specificity of uPA–fibulin-5 binding

(A) Schematic representation of uPA-deletion variants. KD, kringle domain; PD, protease (catalytic) domain. (B) Binding of fibulin-5–c-Myc to uPA-deletion variants. Equimolar amounts of uPA-deletion variants were immobilized on CNBr-Sepharose. Equal aliquots of the resulting affinity matrices were incubated with aliquots of cell culture medium from fibulin-5–c-Myc-expressing CHO cells. The bound proteins were eluted with the sample buffer, and subjected to Western blotting using an anti-c-Myc antibody. A representative result from three independent experiments is shown. (C) Top panel: schematic representation of fibulin-5-deletion variants. EGF1–6, Ca^{2+} -binding epidermal growth factor-like domain; RGD, Arg-Gly-Asp-containing integrin-binding motif; CT48, fibulin-5 variant lacking the C-terminal 48 amino acids (amino acids 400–448); CT, fibulin-5 variant lacking the C-terminal globular domain (amino acids 342–448); EGF1, fibulin-5 variant lacking the N-terminal Ca^{2+} -binding EGF-like domain which contains the RGD motif (amino acids 41–68). Bottom panel: HEK-293 cells were transfected with vectors encoding each fibulin-5-deletion variant for 48 h. Equal amounts of each lysate were separated using SDS/PAGE and Western blotting and the blots were probed with an anti-V5 antibody. (D) Binding of the V5-tagged fibulin-5-deletion variants to immobilized uPA. HEK-293 cells were transfected with the vectors encoding the fibulin-5-deletion variants shown in (C). Equal amounts of each lysate, diluted to contain equal amounts of total protein, were applied to BSA-Sepharose or scuPA-Sepharose. Bound proteins were eluted with sample buffer, subjected to Western blotting and probed with an anti-V5 antibody. A representative result from three independent experiments is shown. (E) Binding of V5-tagged fibulin-5 to immobilized scuPA. scuPA-Sepharose or BSA-Sepharose was incubated

in the absence or presence of 0.1 μ M plasmin for 10 min at 37 °C and the reaction was stopped by the addition of aprotinin (100 k-units/ml). Matrices were incubated with the cell culture medium of HEK-293 cells, transfected with pCEP4-FBLN5 for 1 h and washed with PBS supplemented with 2 mM CHAPS, PBS supplemented with 0.85 M NaCl and 2 mM CHAPS, and again with PBS supplemented with 2 mM CHAPS. Samples were mixed with non-reducing SDS/PAGE sample buffer, heated for 5 min at 60 °C and analysed by immunoblotting using an anti-His antibody. **(F)** Binding of the V5-tagged fibulin-5 to immobilized tcuPA in a solid-phase assay. Plates (96-well) were coated with 35 μ g/ml recombinant tcuPA or BSA, blocked with DPBS supplemented with 1 % BSA and incubated with fibulin-5–V5–His for 1 h. Bound fibulin-5–V5–His was detected using HRP-conjugated anti-V5 antibody, followed by development with 3,3',5,5'-tetramethylbenzidine. Absorbance at 450 nm was measured using a microplate reader. **(G)** Binding of fibulin-5–c-Myc to immobilized scuPA in the presence or absence of plasminogen. Medium from fibulin-5–c-Myc-expressing CHO cells was incubated with immobilized uPA in the presence or absence of a 10-fold excess of plasminogen and/or 100 k-units/ml aprotinin and 0.5 mM PMSF. Bound proteins were eluted and analysed by Western blotting using an anti-c-Myc antibody. For Western blots, the molecular mass in kDa is indicated on the left-hand side.

Figure 3. Fibulin-5 forms complexes with uPA-uPAR and is required for uPA chemotactic activity

(A) Immunodetection of uPA and fibulin-5 on the surface of the PSMCs. PSMCs were fixed with 4 % paraformaldehyde and fibulin-5 was visualized using rabbit polyclonal (top panel) and monoclonal (bottom panel) antibodies and Alexa Fluor® 488-conjugated goat anti-rabbit or Alexa Fluor® 555-conjugated goat anti-mouse secondary antibodies. uPA was visualized using either monoclonal (top panel) or polyclonal (bottom panel) antibodies and the secondary antibodies as described above. The nuclei were counterstained using Hoechst dye. F-actin (filamentous actin) was visualized using Alexa Fluor® 647-conjugated phalloidin and is shown pseudocoloured white. Total mouse and rabbit IgG were used as negative controls and displayed no staining (results not shown). Scale bar, 20 μ m. (B) uPA stimulates directed MEF migration. *Fbln5*^{-/-} MEFs or *Fbln5*^{+/+} MEFs were placed in

DMEM containing 0.5 % serum into the upper chamber of the Transwell. DMEM containing 0.5 % serum or DMEM containing 0.5 % serum and 25 nM mouse scuPA were added into the bottom wells. Cells were allowed to migrate through the pore membrane of the Transwell for 4.5 h, fixed and migrated cells were counted. Values are means $\pm$ S.D. from four independent experiments performed in duplicate. $*P < 0.05$. **(C)** uPA stimulates random MEF migration in the scratch assay. Confluent *Fbln5*^{-/-} MEFs or *Fbln5*^{+/+} MEFs were incubated in DMEM containing 0.5 % FBS for 16 h, scratched with a P10 pipette tip and incubated with 25 nM mouse scuPA or vehicle. Images were acquired every 30 min over a period of 14 h using a Leica AF 6000LX microscope, and the rate of cell migration was calculated by measuring the reduction in the scratched area in every track every 30 min. Values are the means $\pm$ S.D. from three independent experiments performed in triplicate. $***P < 0.001$. **(D)** uPA stimulates MEF migration in an-uPAR-independent manner. *Fbln5*^{-/-} MEFs or *Fbln5*^{+/+} MEFs in DMEM containing 0.5 % serum were placed into the upper chamber of the Boyden chamber and incubated for 4 h. Chemotactic activity is expressed as the fold stimulation relative to the basal migration in the absence of uPA or FBS. Values are means $\pm$ S.D.; $*P < 0.05$ and $***P < 0.001$ compared with controls, $n = 4$. ns, not significant.

Figure 4. Mechanistic requirements of fibulin-5-dependent migration in response to uPA
 (A and B) Exogenously added recombinant fibulin-5 restores the ability of *Fbln5*^{-/-} MEFs to migrate in response to uPA. *Fbln5*^{-/-} MEFs were pre-loaded with calcein, trypsinized, placed in DMEM containing 0.5 % serum, pre-incubated with recombinant fibulin-5 or vehicle (200 nM) for 1 h, and then placed into the upper chambers of the Boyden chamber for 4 h. uPA or control medium was placed in the lower chambers. Chemotactic activity is expressed as (A) relative fluorescence, which reflects the relative number of cells, or (B) fold stimulation relative to basal migration in the absence of uPA. Values are means ± S.D.; ***P* < 0.01, ****P* < 0.001; *n* = 3. (C and D) *Fbln5*^{RGE/RGE} MEFs do not migrate in response to uPA. Wild-type *Fbln5*^{wt/wt} MEFs and *Fbln5*^{RGE/RGE} MEFs were prepared, subjected to

chemotactic stimuli and motility was quantified as described in (A) and (B). Values are means $\pm$ S.D.; * $P < 0.05$; *** $P < 0.001$; $n = 3$. (E) Catalytic activity of uPA and $\beta 1$ -integrins are required for uPA-dependent migration of PSMCs. PSMCs were starved for 18 h in Medium 231 supplemented with 1 % (v/v) FBS, then preloaded with calcein, and detached as described in (A). Cell suspensions of equal density were pre-incubated either with normal mouse IgG or mouse-blocking anti-(human $\beta 1$ -integrin) (10 μ g/ml) antibodies for 1 h and then placed in the upper chambers of the Boyden chamber. Human wild-type scuPA or (S356A)scuPA (25 nM each) was added to the lower chamber. Cell transmigration through the filter was assessed as described in (A). Values are means $\pm$ S.D.; * $P < 0.05$, *** $P < 0.001$; $n = 3$. (F and G) Aprotinin inhibits migration of *Fbln5*^{+/+} MEFs in response to uPA. Wild-type MEFs were subjected to chemotactic stimuli as described in (A) alone or in the presence of aprotinin (100 k-units/ml) and transmigration was assessed as described above in (A) and (B) respectively. Values are means $\pm$ S.D.; * $P < 0.05$; *** $P < 0.001$; $n = 3$. ns, not significant.

Figure 5. uPA mediates cleavage and dissociation of fibulin-5 from β 1-integrin

(A) Association of fibulin-5 with the β 1-integrin subunit in cells left untreated or incubated with uPA. HEK-293 cells transfected with the pCEP4-FBLN5 vector were incubated in the presence or absence of 25 nM scuPA for 4 h. The cells were lysed, and protein complexes with the β 1-integrin subunit were immunoprecipitated from the lysates. Immunoprecipitated proteins were subjected to SDS/PAGE and Western blotting using anti-V5 antibodies conjugated with HRP to detect co-immunoprecipitated V5-tagged fibulin-5. (B) Incubation of cells with uPA induces cleavage of fibulin-5. HEK-293 cells, transfected with pCEP4-FBLN5, were treated as described in (A), and cell culture medium was subjected to SDS/PAGE and Western blotting to detect V5-tagged fibulin-5. (C) β 1-Integrin co-localizes with fibulin-5 in untreated, but not in uPA-exposed, PSMCs. PSMCs plated in chamber-slides were starved as in Figure 4(E), incubated with 25 nM scuPA for 4 h and fixed as described in Figure 3(A). Fibulin-5 was visualized as described in Figure 3(A) using a monoclonal anti-fibulin-5 antibody and Alexa Fluor[®] 555-conjugated goat anti-mouse antibodies (red), β 1-integrin was visualized using a rabbit polyclonal anti-(β 1-integrin) antibody and Alexa

Fluor[®] 488-conjugated goat anti-rabbit antibodies (green). **(D)** Overexpression of a non-cleavable mutant of fibulin-5 inhibits migration of PSMCs in response to uPA. PSMC were transduced either with 'empty' lentivirus or lentivirus encoding non-cleavable human fibulin-5-c-Myc. At 24 h later PSMC chemotactic activity in response to scuPA was assessed as described in Figure 4(A). Values are means $\pm$ S.D.; * $P < 0.05$; $n = 3$. ns, not significant.