PTEN expression in endothelial cells is down-regulated by uPAR to promote angiogenesis

Matthias Unseld[1]; Anastasia Chilla[1]; Clemens Pausz[1]; Rula Mawas[1]; Johannes Breuss[2]; Christoph Zielinski[1]; Gernot Schabbauer[3]; Gerald W. Prager[1]

[1] Department of Medicine I, Comprehensive Cancer Center, Medical University of Vienna, Vienna, Austria; [2] Institute of Vascular Biology and Thrombosis Research, Medical University of Vienna, Vienna, Austria; [3] Institute for Physiology, Medical University of Vienna, Vienna, Austria

Summary

The tumour suppressor phosphatase and tensin homologue (PTEN), mutated or lost in many human cancers, is a major regulator of angiogenesis. However, the cellular mechanism of PTEN regulation in endothelial cells so far remains elusive. Here, we characterise the urokinase receptor (uPAR, CD87) and its tumour-derived soluble form, suPAR, as a key molecule of regulating PTEN in endothelial cells. We observed uPAR-deficient endothelial cells to express enhanced PTEN mRNA- and protein levels. Consistently, uPAR expression in endogenous negative uPAR cells, down-regulated PTEN and activated the PI3K/Akt pathway. Additionally, we found that integrin adhesion receptors act as trans-membrane signaling partners for uPAR to repress PTEN transcription in a NF-κB-dependent manner. Functional *in vitro* assays with endothelial cells, derived from uPAR-deficient and PTEN heterozygous crossbred mice, demonstrated the impact of uPAR-dependent PTEN regulation on cell motility and survival. In an *in vivo* murine angiogenesis model uPAR-deficient PTEN heterozygous animals increased the impaired angiogenic phenotype of uPAR knockout mice and were able to reverse the high invasive potential of PTEN heterozygots. Our data provide first evidence that endogenous as well as exogenous soluble uPAR down-regulated PTEN in endothelial cells to support angiogenesis. The uPAR-induced PTEN regulation might represent a novel target for drug interference, and may lead to the development of new therapeutic strategies in anti-angiogenic treatment.

Keywords

Angiogenesis, endothelial cell behaviour, PTEN, urokinase receptor

Correspondence to:
Gerald W. Prager
Department of Medicine I
Comprehensive Cancer Center Vienna
Medical University of Vienna
1090 Vienna, Austria
Tel.: +43 1 40400 4450, Fax: +43 1 40400 4451
e-mail: gerald.prager@meduniwien.ac.at
or
Gernot Schabbauer
Institute for Physiology
Center for Physiology and Pharmacology
Medical University of Vienna
1090 Vienna, Austria
Tel.: +43 1 40160 31427, Fax: +43 1 40160 93101
E-mail: gernot.schabbauer@meduniwien.ac.at

Financial support:
This study was supported by the Initiative Krebsforschung and grants from the Austrian Science Foundation (FWF: P23199 to G. W. P. and P23730, P24802 and SFB54-InThro-P10 to G. S.).

Received: January 13, 2015
Accepted after minor revision: March 16, 2015
Epub ahead of print: April 30, 2015

http://dx.doi.org/10.1160/TH15-01-0016
Thromb Haemost 2015; 114: 379–389

Introduction

Angiogenesis is a tightly regulated process when the balance of pro- and anti- angiogenic molecules is tipped to favour the outgrowth of new blood vessels from preexisting vasculature (1). Major pro-angiogenic molecules, such as members of the VEGF-family, induce signalling cascades to activate endothelial cells leading to up-regulation of protease expression (2). Subsequently, cell migration, cell survival and cell proliferation are up-regulated via activation of the major MAPK- and PI3K/Akt pathways (2–4). The phosphatase and tensin homologue (PTEN) is the central inhibitor of the PI3K–pathway and is frequently found to be mutated or lost in multiple tumour types (5, 6). Additionally PTEN was described in endothelial cells as a major regulator of angiogenesis and was proposed to act via the PI3K/Akt and integrin signalling pathway (6, 7).

The impact of PTEN regulation in the tumour microenvironment on tumourigenesis and tumour-propagation was described recently as expression of PTEN centrally affects cell behaviour of fibroblasts as well as endothelial cells (8–10). High levels of PTEN were shown to inhibit VEGF-induced sprouting and capillary tube formation (11). Consistently, cells with functionally impaired PTEN showed enhanced proliferative and migratory endothelial cell activity, while increased vascular sprout formation was observed in an aortic ring assay whenever PTEN levels were decreased (10, 11). Knock-out of the PTEN gene in endothelial cells

was embryonic lethal in mice at day E10.5 due to bleeding complications, while heterozygous PTEN (+/-) endothelial cells showed enhanced expression of angiogenic molecules, thereby indicating the relevance of PTEN for normal vascular development (10). *In vivo,* PTEN down-regulation increased angiogenesis in a matrigel plug assay as well as in Lewis Lung Carcinoma-based xenografts (11). Although a large amount of data determine a role of PTEN in tumour-angiogenesis, the mechanism, which regulates PTEN expression in endothelial cells, is poorly understood.

We and others have recently described the functional role of the urokinase receptor (uPAR, CD87) in growth factor-induced endothelial cell activation (12–16). The GPI-anchored protein uPAR is up-regulated upon endothelial cell activation and is overexpressed in many tumour cells (17, 18). The receptor is known to induce intracellular signal transduction via interacting with transmembrane partner molecules, such as LRP-family members or integrin-adhesion receptors (12, 17, 18). Thus, we observed that uPAR was essential for effective endothelial cell migration and invasion when induced by major angiogenic growth factors (13). Also the soluble form of uPAR, suPAR, supports endothelial cell motility and was suggested to support tumour-angiogenesis (19, 20). Consistently, suPAR acts as a worse prognostic clinical marker in various cancer types, such as those deriving from pancreas, colon or ovary (21–23).

In our current study, we found that overexpression of uPAR led to its interaction with integrin adhesion receptors to interfere with PTEN transcription in an NF-κB dependent manner. As a consequence Akt-dependent cell behaviour, such as cell motility and survival *in vitro* and endothelial cell invasion *in vivo,* was promoted whenever uPAR suppressed PTEN expression.

Materials and methods

Extended description of *Materials and methods* is available in the Supplementary Material online at www.thrombosis-online.com. The description includes detailed information about Tissue culture, Immunoblotting, RNA isolation, RT-PCR, qRT-PCR, fluorometry, trans-migration assay, survival assay, aortic ring assay, modified *in vivo* angiogenesis assay, immunohistochemistry and mouse genotyping as described by us before (12, 13, 24, 25).

Animal studies

All animal experiments were performed according to the Austrian animal experimentation law, the ethical guidelines and good scientific practice guidelines of the Medical University of Vienna and were approved and comply with institutional guidelines (BMWF-66.009/0241-II/3b/2011).

A detailed description of murine background was published recently (10, 13, 25). Floxed PTEN mice were kindly provided by Tak W. Mak (University of Toronto, ON, Canada). Tie2 cre mice were kindly provided by Randall S. Johnson (UCSD, San Diego, CA, USA). Intercrossed mice were backcrossed to a C57BL/6J background for at least ten generations. uPA -/-, uPA-/- and PAI-1 -/- mice were kindly provided in 25% 129S/v: 75% C57BL/6 background by Dr. Peter Carmeliet (Center for Transgene Technology, Leuven, Belgium). Mice were backcrossed for at least eight generations. Littermate-controlled experiments were performed using 8– to 12-week-old male mice.

Endothelilal cell isolation

Endothelial cells were isolated according to Fehrenbach et al. (26) with some modifications: Briefly, magnetic beads (Dynabeads, Invitrogen, CA, USA) were incubated with anti-CD31 antibody (AbDSerotec, Oxford, UK) overnight a day before the experiment at 4°C. At the day of the experiment, lung vessels were flushed via injecting DMEM into the vena cava. Consequently, 1ml of 1.5 mg/ml Collagenase A (Roche diagnostics, Basel, Switzerland) was injected into the trachea. Lungs were removed, dissected and transferred into a 50-ml tube filled with 5 ml Collagenase A. The suspension was incubated for 30 minutes (min) and agitated every 5 min in a water bath at 37°C. Following incubation, 15 ml 1xPBS were added to the lung-collagenase suspension. Tubes were shaked for 30 seconds and filtered through a 70 μm cell strainer under sterile conditions. Tubes were centrifuged (5 min, 1,500 rpm) and cells were transferred into the magnetic beads/CD31 suspension with serum containing medium for 20 min at room temperature. Following incubation, magnetic beads were washed four times with HBSS + 5% FBS. Consequently, cells were seeded on gelatin-coated flasks and filled with M199 endothelial cell complete medium containing endothelial cell growth supplement (ECGS, Technoclone, Vienna, Austria). A second purification step was performed after four days to increase endothelial cell purity. FACS analysis was performed to verify endothelial cell purity.

Preparation of soluble uPAR

Protein G Agarose beads (Pierce, Thermo Scientific Inc., Rockford, IL, USA) were incubated with rabbit α- mouse uPAR antibody (Fl-290, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight. In parallel, LLC-1 cells (Lewis lung carcinoma) were seeded in serum-free medium and supernatant was collected after 48 hours (h). Medium was centrifuged (5min, 210g) and supernatant was concentrated using ultra centrifugal tubes (25 min, 4,000g; molecular weight cut-off 10×10^3Da, Amicon®, Millipore, Billerica, MA, USA). Subsequently, anti-uPAR beads were resuspended with concentrated conditioned medium and incubated overnight. Following incubation, uPAR was eluted from beads using Glycin/HCl (0.1 m; PH 2,8; 5 min) and mixed with TRIS buffer to adjust PH according to the manufacturer's protocol. Sample was mixed with PBS and again centrifuged (4,000g, 25 min) to clean sample from residues. Soluble uPAR concentration was measured by ELISA (antibodies-online Inc., Aachen, Germany). uPAR purity was verified performing MALDI-analysis using an AXIMA-CFRplus (Shimadzu, Manchester, UK).

Plasmid constructs and transfection

HEK293 cells were transfected with a neomycin resistance pcDNA3.1 plasmid (addgene, Cambridge, MA, USA) with encoding for full-length uPAR. Electroporation was performed via AmaxaNucleofector using Nucleofector Kit V (Lonza, Basel, Switzerland) according to the manufacturer`s protocol. Subsequently, stable transfected cells were selected by G418 (Calbiochem, Darmstadt, Germany) in DMEM medium (Gibco, Carlsbad, CA, USA).

Scratch assay

96-well plates were coated with 1% Gelatine overnight. A total of 20,000 cells were added to each well and incubated at 37°C. Cells were starved in 5% FBS the day before the experiment. The assay was performed under serum free conditions (1% BSA) after cells were washed twice with PBSx1. Consequently, a scratch wound was made using the Essen Wound Maker (EssenBioScience Inc., Ann Arbor, MI, USA), a device to assure equal scratches in all wells. The cells were washed again three times with PBSx1 to remove detached cells. After the washing steps, serum-free medium containing 1%FBS with or without 50 ng/ml VEGF (promocell, Heidelberg, Germany) was added to the murine endothelial cells and incubated for 24 h. Pictures were taken every hour using the IncuCyte Zoom® (EssenBioScience Inc.), a microscopic camera in an incubator, which allows live-cell imaging during incubation. Scratch closure was analysed after 24 h by the systems automated analysis software and verified by measuring the wound area using ImageJ (Version 1.45s, NIH, Bethesda, MD, USA).

Statistics

Calculated values are expressed as mean ± SD or as indicated. Statistical significances were determined by unpaired Student's t-test. Significance was assigned to a value of *p< 0.05, **p< 0.005 and

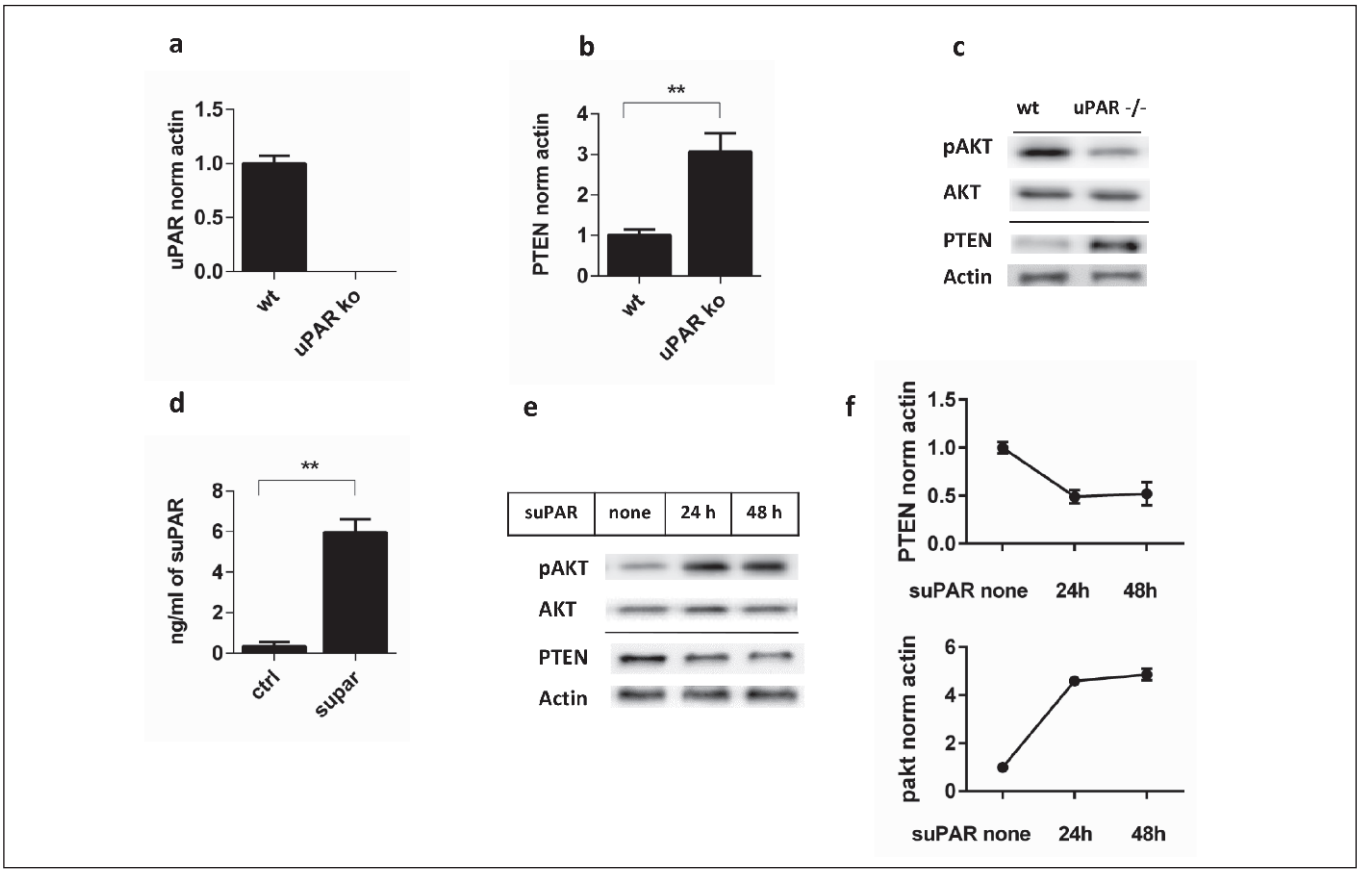

Figure 1: The urokinase receptor affects PTEN levels in endothelial cells. A-C) Murine lung endothelial cells were isolated from either wild-type or uPAR-deficient mice and cell purity was verified by fluorometric analysis (Suppl. Figure 1, available online at www.thrombosis-online.com). PTEN (B) mRNA- and (C) protein levels were assessed performing (B) qPCR and (C) Western blot analysis. Relative mRNA expression levels of PAR (A) and PTEN (B) were normalised to GAPDH. D-F) Soluble uPAR was derived from LLC-1 cell supernatant and purified by uPAR pull down. Specificity of immunoprepared protein was analysed performing MALDI. D) Soluble uPAR concentrations of suPAR depleted control (ctrl) and conditioned medium containing soluble uPAR (5.97 ± 0.64 ng/ml) were quantified by ELISA. E) uPAR deficient endothelial cells were incubated with either suPAR depleted conditioned control medium (none) or conditioned medium containing suPAR at different time points. PTEN, phosphoAKT and panAKT levels were determined by Western blot. F) Densitometry was performed using AlphaEaseFC-program to semi-quantitative analyse PTEN protein levels normalised to respective actin stains. n=4. Error bars, SD; p-values based on Student`s t-test; ** p <0.005.

***p< 0.0005. Results were reproduced in three independent experiments.

Results

The presence of uPAR determines PTEN levels in murine endothelial cells

In previous studies we found that uPAR deficient mice showed decreased vessel ingrowth into VEGF embedded matrigel when compared to respective C57BL/6 control mice (12, 13). Here, we confirmed these data by using matrigel filled angioreactors (►Figure 5). Detailed analysis of the underlying mechanism revealed that uPAR`s role in endothelial cell behaviour was not limited to its role as serine-protease receptor: characterisation of murine lung endothelial cells, isolated from either uPAR -/- or control wild-type C57BL/6 mice, showed enhanced PTEN mRNA- as well as protein expression when uPAR was absent as revealed by qRT-PCR and Western blotting, respectively (►Figure 1A-C). Consistently, AKT phosphorylation was decreased upon PTEN overexpression in uPAR deficient endothelial cells (►Figure 1C). Endothelial cell isolation from respective mice was supported by CD31 immuno-sorting and confirmed by fluorometric analysis (Suppl. Figure 1, available online at www.thrombosis-online.com). When uPAR-deficient murine endothelial cells, however, were stimulated with tumour-secreted immuno-purified soluble uPAR (5.97 ± 0.64 ng/ml; ►Figure 1D), which was derived from Lewis lung carcinoma cells (LLC-1), PTEN levels were significantly decreased, whereby uPAR depleted supernatant was ineffective to influence PTEN (►Figure 1E). The maximum of PTEN suppression on suPAR stimulation was reached after 24 h and was sustained for at least 48 h. MALDI technology verified glycosylated suPAR forms of 44 to 53 kDa as the biological active compound on PTEN regulation (27, 28). Consistently, AKT activation was observed in the presence of suPAR with low PTEN levels (►Figure 1E, F). Both, uPAR and PTEN, are central regulators of angiogenesis, but a functional link between these molecules has so far not being demonstrated. As in the absence of uPAR increased PTEN mRNA-

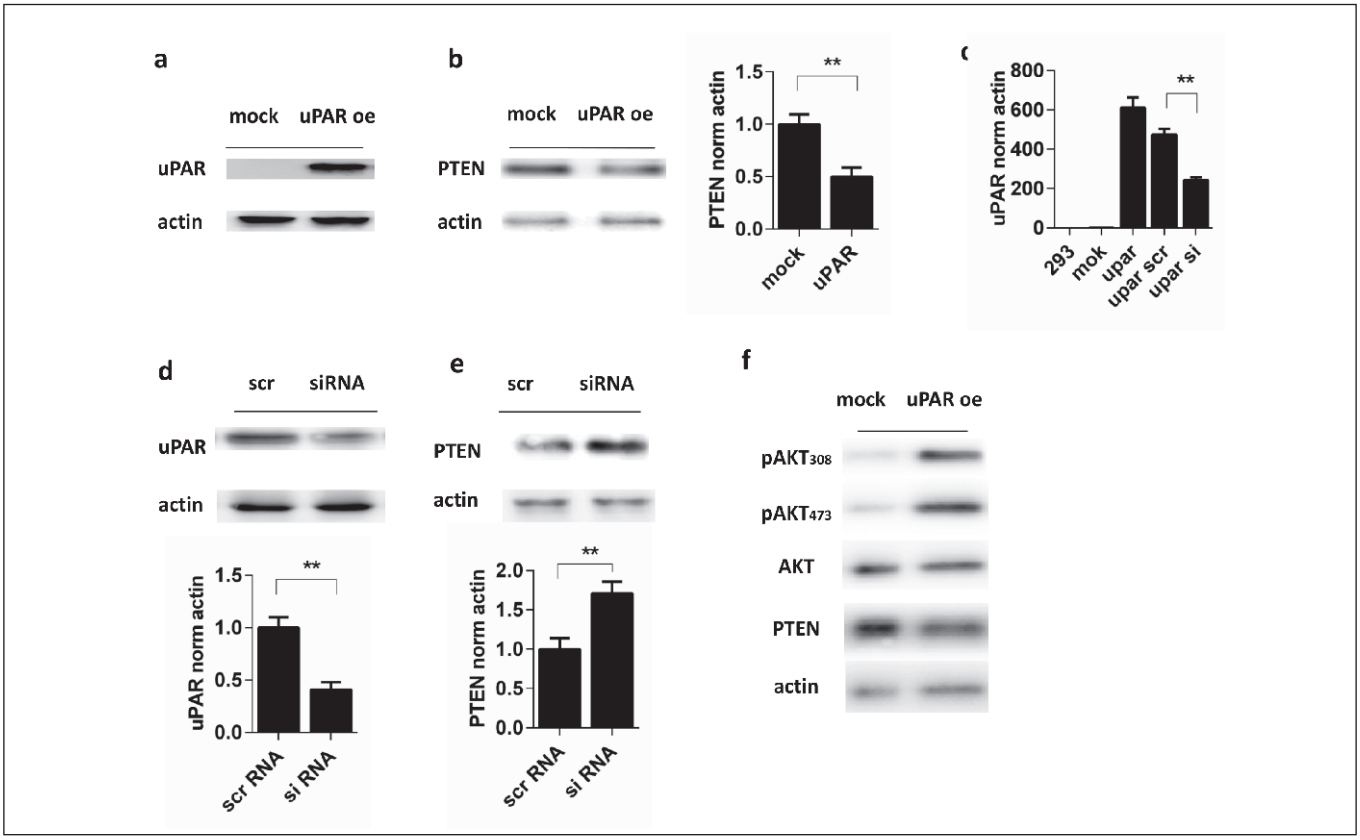

Figure 2: uPAR expression regulates PTEN levels and AKT activation. A) Endogenous negative uPAR HEK cells were stable transfected with a control vector (mock; pcDNA3.1) or a human uPAR expressing vector (HEK uPARoe; pcDNA3.1). Western blotting was performed to confirm uPAR protein expression; n=4. B) Cell lysates from either mock transfected control (mock) – or uPAR expressing HEK cells (uPARoe) were analysed for PTEN protein levels via Western blotting. Densitometry was performed to semi-quantitative analyse PTEN protein levels normalised to respective actin immunostains; n=4. C-E) HEK uPAR expressing cells were transfected with scrambled control or siRNA against uPAR (validated predesigned siRNA; Ambion; Sense 5–3:CCGGAGUUAUACCGUAAGAtt). Silencing efficiency was verified via detecting uPARmRNA (C) as well as protein (D) levels; n=3. E) Changes in PTEN protein levels were analysed by Western blot following siRNA mediated silencing of uPAR, normalised to actin and analysed via densitometry; n=3. F) Western blotting was performed to investigate AKT phosphorylation on Thr_{308} and Ser_{473}; n=3. Error bars, SD; p-values based on Student`s t-test; ** p < 0.005.

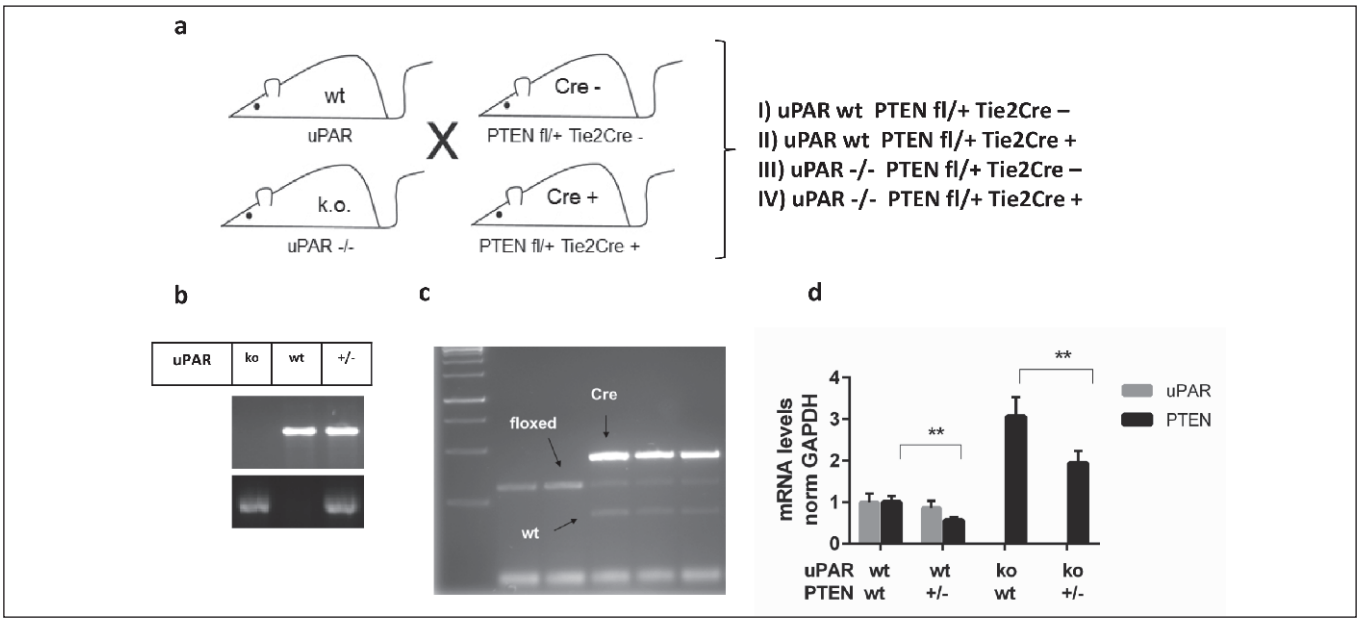

Figure 3: Generation of uPAR-/- PTEN +/- mice. A-C) uPAR-deficient mice were crossbred with PTEN Tie2Creflox (PTEN +/-) mice to receive four different genetic backgrounds. A) Schematic presentation of uPAR -/- and PTEN +/- mice. B) PCR analysis of genomic DNA isolated from uPAR deficient mice. Upper lane indicates uPAR knock out product (0.97 kb); lower lane represents the wild-type product (0.78 kb). C) PCR analysis of genomic DNA isolated from PTEN flox/Tie2cre mice (cre band 500 bp, flox band 335 bp, wt band 228 bp). D) Endothelial cells were isolated and mRNA levels of uPAR and PTEN were determined by qPCR. uPAR levels were not detectable in cells derived from uPAR knock out (uPAR ko) mice. Target genes were normalised to GAPDH; n=3. Error bars, SD; ** $p < 0.005$.

as well as protein levels were found, we next aimed to investigate PTEN levels in an uPAR reconstituted cell model.

uPAR overexpression down-regulates PTEN and activates the PI3K/Akt-pathway

Therefore, we stably transfected uPAR-negative HEK 293 cells with a plasmid (pcDNA3.1) encoding full-length human uPAR or an empty vector as mock control (▶Figure 2A). While uPAR-deficiency led to sustained PTEN expression (▶Figure 1B, C), a decrease in PTEN levels was observed in uPAR over-expressing HEK cells (▶Figure 2B). In mock transfected controls however, PTEN levels were unchanged and comparable to non transfected controls. Furthermore, silencing of uPAR using siRNA (silencing efficiency: 51 ± 5.89%, ▶Figure 2C, D) in stable uPAR expressing HEK cells led to a reconstitution of PTEN protein levels (▶Figure 2E). As a consequence of uPAR-dependent PTEN down-regulation, the main downstream signalling molecule AKT was activated; thus, $pAKT_{Ser473}$ as well as $pAKT_{Thr308}$ levels were found to be up-regulated whenever uPAR was overexpressed (▶Figure 2F). These data indicate that uPAR plays an important role in regulating PTEN levels, which led to a functional signalling consequence on the activation of the PI3K/Akt pathway.

Generation of uPAR -/- PTEN +/- mice

To investigate the biologic role of PTEN in uPAR-induced angiogenesis, we aimed to establish an appropriate *in vivo* system. Therefore, we crossbred mice, heterozygous for PTEN in endothelial cells (Tie2creflox+) (10) with uPAR-deficient mice (13) (▶Figure 3A). For genotyping, murine ear tissue was extracted and PCR was performed as described previously (25) to classify siblings and to confirm previous data about recombination efficiency of Tie2creflox (▶Figure 3B-D) (10). Offspring and endothelial cells, derived from these crossbreds enabled further characterisation of the uPAR-induced PTEN regulation *in vivo* as well as *in vitro*. Littermate-controlled experiments were performed using 8– to 12-week-old male mice with the same genetic background.

Biological impact of genetic uPAR and/or PTEN alteration

To investigate a potential biologic role of uPAR-dependent PTEN regulation in cell motility, we performed an *in vitro* trans-migration-, a wound closure- as well as an *ex vivo* aortic ring assay. Results suggested that in uPAR-deficient endothelial cells, VEGF (50 ng/ml)-induced transmigration was significantly decreased ($p<0.0005$) (▶Figure 4A). This finding is in line with previous observations (10, 11, 13). However, when in uPAR-/- endothelial cells, PTEN was genetically down-regulated (Tie2creflox+) (10), the decreased migratory phenotype was completely reverted to comparable levels as found in unstimulated control cells ($p<0.0005$). Consistently, in a wound closure assay, we found a delayed coalescence of uPAR -/- endothelial cells; an effect which was completely neutralised in PTEN +/-, uPAR -/- endothelial cells (▶Figure 4B; $p<0.0005$). In both assays we were able to confirm

previous data (10–13), which indicated that PTEN reduction (PTEN +/-) led to a remarkable enhanced migratory phenotype.

Finally, we studied capillary outgrowth in an *ex vivo* aortic ring assay (►Figure 4C). After six days of murine $VEGF_{164}$ stimulation, capillary outgrowth was significantly reduced in uPAR -/- aortic rings when compared to wild-type controls. However, this effect was reverted in aortic rings of uPAR -/- PTEN +/- crossbred animals ($p<0.05$).

Similar results were found in endothelial cell survival.

Consistent with the enhanced activation of the Akt-survival pathway in HEK uPAR expressing cells (►Figure 2F), cell survival, upon cell starvation (BSA 1 %, 24 h) or stimulation with the cytotoxic agent camptothecine (3 µM; full medium; 12 h), was decreased in endothelial cells whenever uPAR was absent (►Figure 4D). The presence of the urokinase receptor was dispensable for survival when PTEN expression was low in uPAR wild-type PTEN +/- endothelial cells. Under these conditions endothelial cell survival was significantly increased when comparable to wild-type control cells (►Figure 4D).

However, the urokinase receptor was decisive as shown by cells derived from uPAR -/-, PTEN +/- mice as those cells showed reduced survival when compared to PTEN +/- cells alone (►Figure 4D). From these data we conclude that uPAR exerts a significant impact on PTEN-dependent endothelial cell behaviour *in vitro*.

In vivo angiogenesis in uPAR-PTEN genetic modified animals

To affirm the *in vitro* results obtained in endothelial cells derived from uPAR/PTEN crossbred, we introduced an *in vivo* angio-

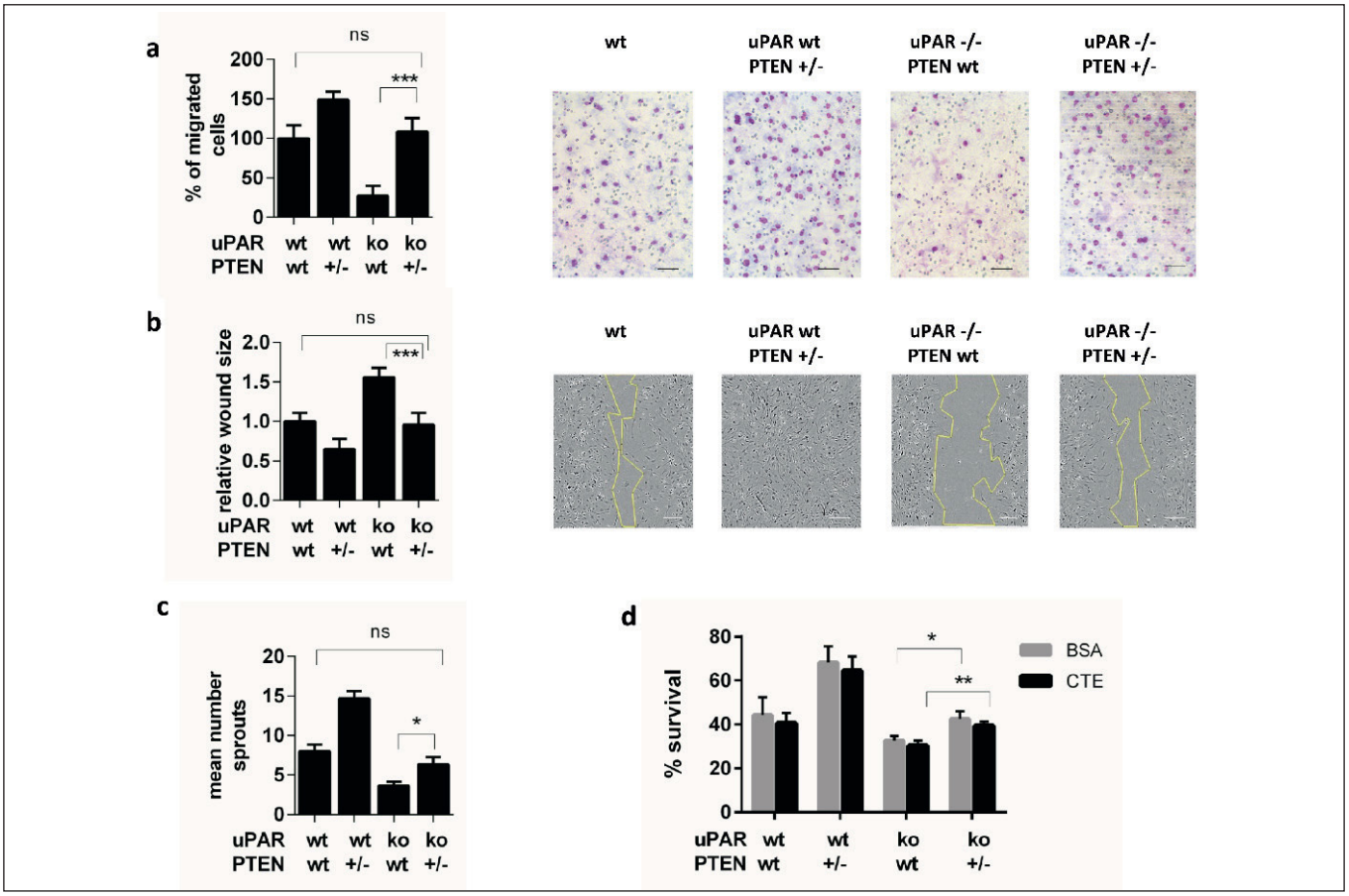

Figure 4: uPAR depletion reverts the pro-angiogenic phenotype of PTEN +/- endothelial cells. Murine endothelial cells were isolated out of i) wt, ii) uPARwt, PTEN +/-, iii) uPAR -/-, PTEN wt, and vi) uPAR -/-, PTEN +/- C57BL/6 mice. A) Cells were allowed to trans-migrate for 24 h through gelatin-coated transwell filters (8 µm pore size) to lower compartments containing M199/5 %BSA + 50 ng/ml mVEGF164. Inserts were fixed, stained with Diff-Quick® staining solution and migrated cells were counted by two independent persons; n=3, scale bars 100 µm. B) Wound healing assay: cell layers were scratched using a special multi-well wound healing device (Essen BioScience Inc.) to prevent direct damage to the coated surface and assure equal scratches in all wells. After flushing with PBSx1 (pH=7.4), cells were supplemented with medium containing 1 %FBS + 50 ng/ml VEGF and were incubated for 24 h. Live cell imaging was performed using the IncuCyte Zoom (Essen BioScience Inc.). Scratch closure was analysed after 24 h by the system`s automated analysis software; n=4, scale bars 200 µm. C) For aortic ring assay, thoracic aorta was intersected in 1 mm rings (n=4 per condition), embedded into matrigel and incubated with M199 containing 50 ng/ml VEGF164 for six days under sterile conditions. Pictures were taken every day and numbers of sprouts were analysed using Image J (NIH). D) Cell survival was assessed upon cell starvation using medium containing 1 % BSA for 24 h (grey bars) or applying the cytotoxic agent camptothecine (3 µM, black bars), incubated with normal full medium for 12 h. Cells were compared to untreated controls. Error bars, SD; p-values based on Student`s t-test; * $p<0.05$, ** $p<0.005$, *** $p<0.0005$.

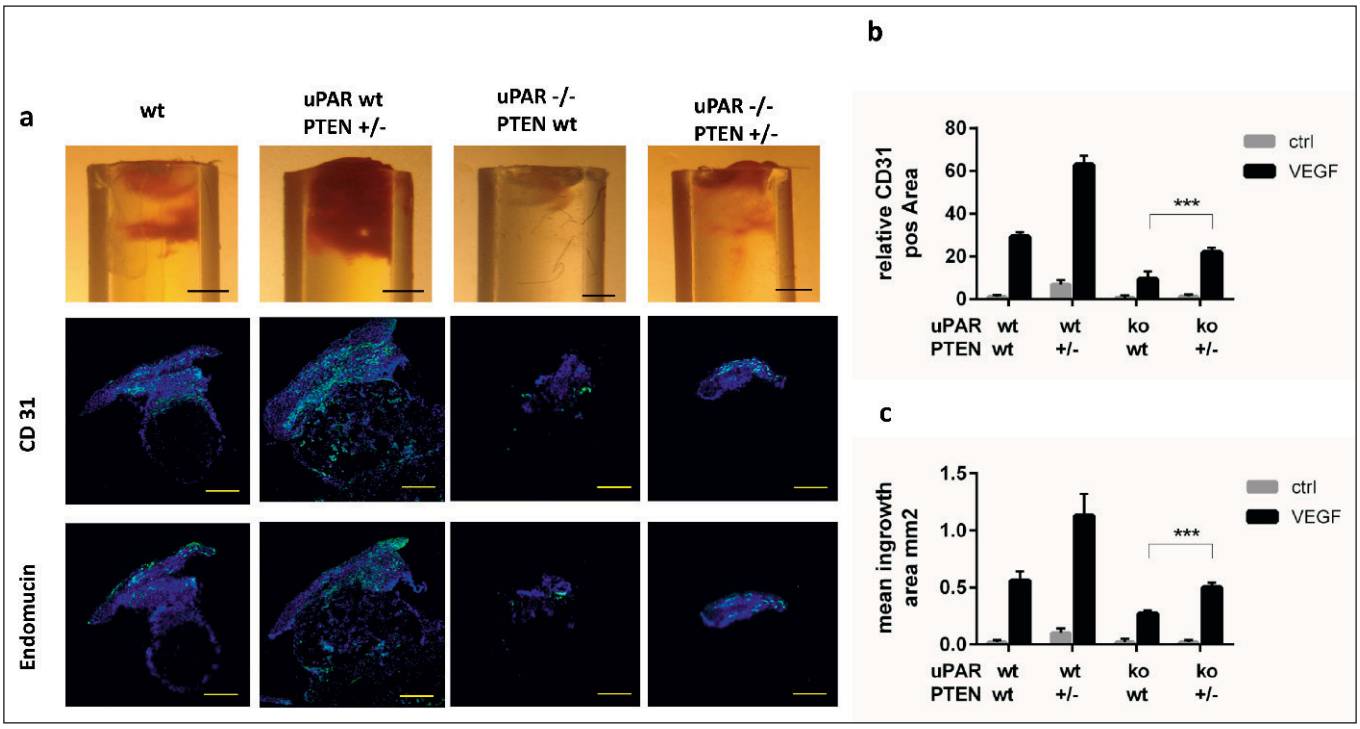

Figure 5: The uPAR/PTEN system regulates angiogenesis *in vivo*. A) The *in vivo* tube invasion assay was performed as described by us earlier (13, 24). Matrigel filled tubes (+ 100 ng/ml mVEGF164) were implanted for 11 days into age- and sex-matched C57BL/6 mice either depleted for uPAR, heterozygous for PTEN, both or neither as indicated. Pictures were taken and IHC was performed using anti-CD31 as well as anti-endomucin antibodies, whereas immuno-reaction was green fluorescent labelled; nuclei were counterstained with DAPI (blue, Suppl. Figure 2, available online at www.thrombosis-online.com); scale bars 500 µm for silicon tubes, 300 µm for IHC, n=8 tubes/group. B) CD31-positive immune-fluorescence staining (green) was automatically quantified normalised to growth factor-depleted control by the computer-assisted software AnalySIS Pro (Soft Imaging System, Münster, Germany). C) CD31 positive ingrowth area (2-dimensional ingrowth area) was determined by Image J. Error bars, SD; p-values based on Student`s t-test; *** $p < 0.0005$.

genesis assays (►Figure 5). By implanted VEGF-embedded matrigel tubes into the flank of respective *i) wt, ii) uPAR wt PTEN +/-, iii) uPAR -/- PTEN wt or vi) uPAR -/- PTEN +/-* mice, endothelial cell invasion was quantified as previously described (13, 24). Consistent with previous data uPAR deficiency led to diminished invasion of endothelial cells into growth factor depleted, but $mVEGF_{164}$ embedded matrigel (12, 13). In addition we observed that genetic down-regulation of PTEN expression (PTEN +/-) in uPAR -/- mice reverted this phenotype. Reduced capillary ingrowth was evident macro-morphologically (0.27 mm^2 ± 0.5 in uPAR -/- compared to 0.49 mm^2 ± 0.51 in uPAR-/- PTEN+/- crossbred mice; ►Figure 5A, B; $p<0.0005$) and was further quantified via anti-CD31– as well as anti-endomucin immunostaining, reflecting endothelial cell positive areas (9.86 ± 3.61 relative CD31 pos. area in uPAR -/- compared to 21.27 ± 2.22 in uPAR-/-, PTEN+/- crossbred mice; $p<0.0005$; ►Figure 5C; Suppl. Figure 2, available online at www.thrombosis-online.com). Notably uPAR deficieny had no effect on endothelial cell proliferation, neither in PTEN +/+ nor in PTEN +/- endothelial cells (Suppl. Figure 8A-B, available online at www.thrombosis-online.com). These data suggest that uPAR-dependent PTEN regulation centrally regulates growth factor-induced endothelial cell invasion *in vivo*.

uPAR affects PTEN via integrin-mediated signal transduction and NF-κB

Finally, we aimed to analyse the molecular mechanism of uPAR-induced PTEN regulation in endothelial cells. We considered a potential uPAR complex formation consisting of its ligand urokinase (uPA), as well as its specific inhibitor the plasminogen activator inhibitor-1 (PAI-1). The ternary complex between uPA/uPAR and PAI-1 was recently identified to be a prerequisite for several uPAR-induced cellular mechanisms, among them, an effective VEGF-induced uPAR internalisation and recycling mechanism (12). We analysed endothelial cells, derived either from uPA-/- or PAI-1 -/- mice, and found that, compared to wild-type control endothelial cells, deficiency of uPA or of PAI-1 neither led to an altered PTEN expression (►Figure 6A). Thus, although uPA/PAI-1/uPAR complex formation is a prerequisite for uPAR recycling, these molecules did not affect uPAR-mediated PTEN regulation. To confirm these observations, we furthermore stimulated HEK uPAR overexpressing cells with active uPA (10 U/ml) in the presence or absence of the uPAR ligand binding blocking antibody R3 (25 µg/ml, Suppl. Figure 3, available online at www.thrombosis-online.com). Again, neither uPA stimulation nor interference with uPAR ligand binding led to alteration of PTEN expression levels.

Next, we were interested whether uPAR/LDL-receptor like molecule interactions were essential for PTEN regulation. Thus, we interfered with uPAR binding to LDL-receptors using the high affinity chaperone receptor associated protein (RAP) in a time- and dose dependent manner (4 to 48 h; 50–500 nmol/l) (12). We observed that addition of RAP had no effect on uPAR-induced PTEN regulation (data not shown). This data indicate that neither uPAR complex formation nor uPAR complex interference with members of the LDLR-family were involved in PTEN down-regulation.

The family of integrin adhesion receptors is a known functional interaction partner of the urokinase receptor (12, 29–31). After blockage of adhesion-induced signal transduction via a cyclic RGD peptide which interferes with αv-integrin ligand binding (32, 33), we found increased PTEN levels in uPAR expressing HEK cells (▶Figure 6B, left panel). As a consequence of enhanced PTEN expression in the presence of cyclic RGD peptides, Akt phosphorylation was decreased. When the inhibitory effect of cyclic RGD was reverted by downstream overexpression of constitutively active FAK (pEGFP-C1, kindly provided by D. Schlaepfer, UCSD [34]), PTEN expression was reduced and Akt phosphorylation reverted (▶Figure 6B left panel, ** p<0.005).This effect was not observed in the absence of uPAR in endogenous uPAR negative HEK cells (▶Figure 6b, right panel, HEK mock). From these data we conclude that uPAR affects PTEN expression via integrin-mediated signal transduction independent of uPA, PAI-1 or LDLR family members.

Furthermore, we analysed whether PTEN regulation upon uPAR is induced via transcription, translation or degradation. Using the proteasome inhibitor MG132 (10 µmol/l) uPAR did not influence degradation of the phosphatase (Suppl. Figure 4, available online at www.thrombosis-online.com). In this context, also XIAP expression induced by uPAR (35), which has been described to act as an E3 ubiquitin ligase for PTEN (36) for its proteasomal degradation, was not involved in uPAR-induced PTEN regulation

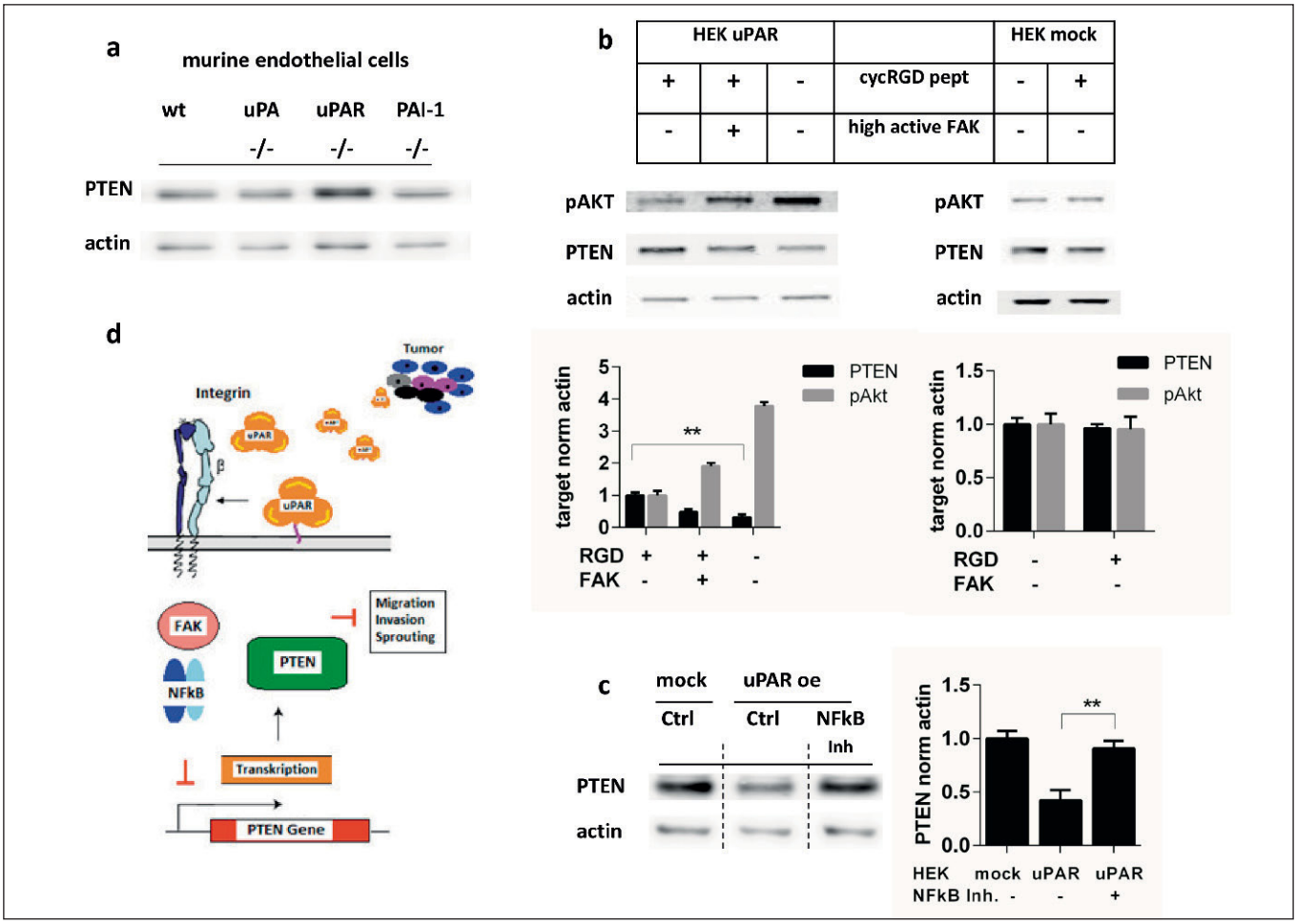

Figure 6: uPAR regulates PTEN levels via integrins independent of uPA/PAI-1. A) Microvascular lung endothelial cells were isolated from either murine wt, uPA-/-, uPAR -/- or PAI-1 -/- C57BL/6 mice and analysed for PTEN protein levels in Western blot analysis; n=3. B) αv-integrins were blocked for 24 h with a specific cyclic RGD peptide (200 nmol/l) in HEK cells either expressing uPAR or respective mock controls. Inhibition of integrin-induced signalling was rescued by transfecting HEK uPAR cells with a constitutive high active FAK vector (pEGFP-C1). PTEN, pAKT473, AKT and actin protein levels were immune-stained in Western blot analysis. Bars represent densitometric analysis of respective protein levels normalised to actin stains; n=3. C) HEK uPAR expressing cells were substituted with 10 µmol/l of the IKK2-inhibitor BMS345541 for 24 h. PTEN levels were assessed via Western blotting and normalised by densitometry to actin stains; n=3. D) Schematic demonstration of the proposed uPAR– PTEN regulation. Error bars, SD; ** p <0.005.

(Suppl. Figure 5, available online at www.thrombosis-online.com). To analyse whether uPAR led to PTEN regulation on the transcriptional or translational level, the transcription inhibitor actinomycin D at a concentration of 5 µg/ml (Suppl. Figure 6, available online at www.thrombosis-online.com) or the translation blocker cycloheximide (10–100 µg/ml, not shown) were applied. As PTEN mRNA levels decreased uniformly in HEK mock control- as well as in HEK uPAR overexpressing cells whenever actinomycin D was present, these results indicate that uPAR-dependent PTEN regulation was not mediated via any effect on mRNA stability, but most likely via regulation of PTEN transcription (Suppl. Figure 6, available online at www.thrombosis-online.com).

To identify the responsible signalling pathway for uPAR/integrin-mediated PTEN regulation, major integrin-activated pathways were analysed. Neither UO126 (25 µmol/l), a MAPK-pathway inhibitor, nor Wortmannin (100 nmol/l) or Ly294002 (10 µmol/l), the PI3-kinase/Akt pathway inhibitors nor pertussis toxin (100 ng/ml), which blocks G protein-coupled receptors (GPCR), showed any effect in uPAR-dependent PTEN regulation (data not shown). Thus, the previous described effect of cleaved uPAR (D2-D3) via an N-terminal SRSRY motif was unlikely (39, 40), which is further supported by the fact that full-length uPAR was detected via MALDI technique. In contrast, BMS 345541 (10 µmol/l), an IKK2 inhibitor described to block the NF-κB pathway (37), significantly abolished the uPAR-induced inhibition of PTEN gene expression (►Figure 6C). This is consistent with previous data, which describe NF-κB as a potent repressor of PTEN transcription via binding of the NF-κB subunit p65 to the phosphatase`s promoter region (38) and our observation that uPAR affects integrin-dependent activation of the IKK2/NF-κB pathway (35). In summary, these data indicate that uPAR, via interacting with integrins, promotes a FAK-dependent signalling cascade and leads to repression of PTEN transcription via the NF-κB pathway.

Discussion

The GPI-anchored protein uPAR is overexpressed in various kind of cancers, among them those deriving form colon, ovary or pancreas (21–23). Thereby, high levels of soluble uPAR have been correlated with poor prognosis (21, 22). We and others have identified uPAR as a central regulator of various steps in angiogenesis (12–16, 39). In growth factor-activated endothelial cells the presence of uPAR is essential for efficient endothelial cell migration and invasion *in vitro* and *in vivo* (12). Cell surface-bound uPAR is distributed to the leading edge of migrating endothelial cells and is localized at focal contacts. Thus, uPAR influences cell motility in functional interaction with integrin adhesion receptors. Furthermore, uPAR promotes endothelial cell survival via integrin-induced signal transduction (35) while endothelial cell proliferation was not affected. Although several endothelial cell functions have been shown to be affected by uPAR, it is hitherto unknown how a GPI-anchored extracellular protein is able to promote several key steps in angiogenesis.

Here, we first report that expression of the major phosphatase PTEN is centrally regulated by the presence of exogenous or endogenous full-length uPAR in endothelial cells. In detail, we found that high levels of the urokinase receptor led to down-regulation of PTEN expression, while low levels or the absence of uPAR led to a constitutively high PTEN expression. By crossbreeding low PTEN expressing PTEN +/- mice with uPAR-deficient animals, we found that the impaired invasive property of uPAR -/- endothelial cells was rescued by normalisation of PTEN levels. That PTEN is a central regulator in angiogenesis was shown before as over-expression of PTEN or dominant-negative constructs of PI3K inhibited angiogenesis, while PTEN +/- endothelial cells showed enhanced neo-vascularisation, tumour- angiogenesis and increased tumour growth (10, 41, 44). Our *in vivo* observations of a PTEN-dependent effect of uPAR-induced angiogenesis were further confirmed by *in vitro* analysis using a migration and wound closure assay. In these particular assays, the impaired migratory phenotype of uPAR deficient endothelial cells, when stimulated by VEGF, was rescued by genetically down-regulation of PTEN. Using established cycloheximide as well as actinomycin D assays, we found that PTEN expression via uPAR was regulated rather on a transcriptional level, but unlikely via induction of mRNA stability or PTEN degradation (Suppl. Figures 4–6, available online at www.thrombosis-online.com). In detail, uPAR led to an integrin αv-dependent activation of FAK to down-regulate PTEN expression in an NF-κB-dependent manner. It was shown previously that the urokinase-system is involved in NF-κB activation via integrins (35). Furthermore, the NF-κB pathway was described to be involved in repression of PTEN transcription (38) and its involvement was confirmed by us using the IKK2 inhibitor BMS-345541, which rescued the uPAR-dependent PTEN down-regulation.

Soluble uPAR contains intact domains, which interact with various molecules to influence intracellular signalling (19, 21, 22). Cleaved uPAR (D2-D3) was demonstrated to induce chemotaxis via its N-terminal SRSRY motif inducing G-protein coupled receptor activation (39, 40). Furthermore, Rao et al. have recently demonstrated that full-length suPAR is capable to induce endothelial cell motility, thus, supporting tumour angiogenesis (19). Our data indicate that soluble uPAR leads to down-regulation of PTEN in endothelial cells (►Figure 1D-F). Although not shown here, these data suggest that high suPAR expressing tumours might activate endothelial cells in their microenvironment, thus contributing to tumour angiogenesis. Respective *in vivo* experiments are currently performed in a consecutive research project.

That non genomic alterations of PTEN expression levels are sufficient to affect tumourigenesis was described recently, although the mechanism was poorly understood so far (42, 43). Our findings provide a mechanism of PTEN regulation in endothelial cells independent of genetic mutations or promoter methylation (42, 43). Overexpression of endogenous uPAR or paracrine stimulation with tumour-derived soluble uPAR, leads to sustained inhibition of PTEN expression in endothelial cells. In conclusion, we have discovered a crucial role for uPAR during angiogenesis, demonstrating that uPAR-induced PTEN regulation enhances a pro-angiogenic endothelial cell behaviour. Thus, interfering with

What is known about this topic?

- Angiogenesis is an essential mechanism in health and disease and its understanding is an important pre-requisite for anti-cancer treatment.
- The tumour suppressor PTEN significantly influences vascular development and tumour angiogenesis.
- uPAR is a major regulator of angiogenic growth factor-induced endothelial cell migration and survival.

What does this paper add?

- The presence of endogenous and exogenous uPAR inversely correlates with PTEN levels.
- Integrins/FAK-NF-κB repress PTEN transcription upon uPAR interaction to influence endothelial cell behaviour.
- PTEN-dependent angiogenesis is crucially regulated by the presence of the urokinase receptor *in vitro* and *in vivo*.

tumour-derived soluble uPAR might constitute a future target for blocking angiogenesis in cancer.

Acknowledgements

We thank G. Hoyer-Hansen for providing αR3. P. Uhrin for supplying uPAR deficient mice; G. Stübinger for assisting with MALDI.

Conflicts of interest

None declared.

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