MATERIALS & METHODS

Cells & culture conditions

Rat aortic endothelial cells (RAEC) were derived from adult male Sprague-Dawley rat (Charles River Laboratories, France) aortic endothelium and characterized as described previously \(^1\). RAECs of passage 2 to 6 were seeded in 150 mm culture dishes at an initial density of \(4.0 \times 10^6\) in DMEM complemented with 10% fetal calf serum, 1% sodium pyruvate, 1% non-essential amino acids, 1% penicillin-streptomycin. At 70% confluence, cells were washed twice in PBS and starved in serum-free DMEM for 30 h.

Tsc2 defective MEFs were obtained from Michael Hall (Biocenter, Basel, Switzerland), HIF-1\(\alpha\) knockouts from Max Gassmann, Clinic for Small Animal Internal Medicine, University of Zürich, Switzerland. Following inhibitors were used: 5 -500 nM rapamycin for mTOR, 0.5 – 2 µM Akt IV inhibitor for Akt (Calbiochem).

Angiogenesis assay in vitro of endothelial spheroids

Endothelial cell spheroids of defined cell number were generated as described elsewhere \(^2\). The spheroid containing fibrin bilayer \(^3\) was overlaid with serum-free DMEM. Rapamycin (20 nM) was added to cultures prior to a 24 h incubation under 21% or 1% O\(_2\). Sprouts emerging from spheroids were photographed digitally on an inverted light microscope (Olympus).

si- and shRNA silencing and transfection

Predesigned siRNAs against rat mTOR (frap1_3 siRNA) were purchased from Qiagen. shRNA containing vectors against raptor and rictor are pKDM-132, a pSuper.gfp/neo-based (Oligoengine) siRNA expressing plasmid targeting ctgtgaactagcacttcag in rictor mRNA; pKDM-162 is a pSuper.gfp/neo-based plasmid
targeting ggacaacggccacaagtac in raptor mRNA. RAEC were transfected with si/shRNA by nucleofection using the basic endothelial transfection kit. Briefly, cells were trypsinized, counted and 10E6 cell per cuvette were mixed with 3µg of si/shRNA and nucleofected by program T27 on a Amaxa nucleopator. Cells were seeded and used 24 h later for experiments. Ivan B&uum (Novartis, Basel, Switzerland) provided HA-mTOR cDNA containing pBABE-Puro retroviral vectors necessary for MEF infection.

**Cell proliferation assay**

To determine cell numbers, 1000 cells/well were seeded into 96-well plates and after 24 h the normal culture medium was replaced with serum-free DMEM. Inhibitors were added 30 min before cultures were incubated for 24 h at 21% O&sub 2 and at 1% O&sub 2 in octuplicates. After 24 h, cell numbers were assessed using Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals) according to the manufacturer's specifications.

**Immunoblotting**

Total cell lysis was prepared with RIPA buffer as described before 1. Nuclear lysates were prepared as described elsewhere 4. After SDS-PAGE, proteins were transferred onto Polyvinylidene fluoride (PVDF) membrane (Millipore). The membrane was blocked with 4% skim milk powder in TBS-Tween solution or 4% BSA and probed with following antibodies: primary chicken polyclonal anti-HIF-1&alpha; (provided by Max Gassmann, Clinic for Small Animal Internal Medicine, University of Zürich, Switzerland), polyclonal anti-raptor and anti-rictor (by Markus A. Rüegg, Biocenter Basel, Switzerland), polyclonal anti-S6K1, polyclonal anti-phospho- S61 Kinase
(Thr\textsuperscript{389}), polyclonal anti-mTOR, polyclonal anti-phospho-mTOR (Ser\textsuperscript{2448} and Ser\textsuperscript{2481}), polyclonal anti-phospho-Akt (Ser\textsuperscript{374}), polyclonal anti-phospho GSK3\(\alpha/\beta\) (Ser\textsuperscript{21/9}), polyclonal anti GSK3\(\beta\), all from Cell Signaling Technology, MA, USA, polyclonal anti-Cyclin D1 and monoclonal anti-p21 (Santa Cruz Biotechnology). HRP-conjugated IgGs (Transduction Laboratories) were used for visualization of relevant proteins on X-ray films by a chemiluminescence reaction (Amersham). Protein bands were analyzed by densitometry and quantified by ImageJ 1.31v software (Wayne Rasband, NIH, USA).

**Immunestaining**

Immunestaining was performed as described previously\(^5\).

**Statistical analysis**

Unless otherwise described, data (mean±SEM) were analyzed for normal distribution (one-way or repeated measures ANOVA), followed by multiple or pairwise comparison with the Bonferroni post test using the program Prism (Graphpad). The number of single experiments compiled is indicated by n. A value of P<0.05 was considered as significant.
References


Supplemental figure legend

Cytotoxicity tests.

RAEC were starved for 30 h and incubated for 24 h at 21% O₂ and 1% O₂ with indicated amount of Akt inhibitor IV. 100% of cytotoxicity was determined by cell lysis with 2% Triton X-100. Akt inhibitor IV concentrations used for proliferation assays are indicated and range in nontoxic doses. Cytotoxicity was measured with the Roche celltoxicity kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland) according to the manufacturer’s specifications.