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Hypoxia-induced endothelial proliferation requires both mTORC1 and mTORC2

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Short title: Hypoxia signaling via mTOR

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Abstract

mTOR is a central regulator of cell growth and has been implicated in responses to stress such as hypoxia. We have shown before that mTOR is required for angiogenesis in vitro and endothelial cell proliferation in response to hypoxia. Here we have investigated mTOR-associated signaling components under hypoxia and their effects on cell proliferation in rat aortic endothelial cells (RAEC).

Hypoxia (1% O₂) rapidly (>30 min) and in a concentration-dependent manner promoted rapamycin-sensitive and sustained phosphorylation of mTOR-Ser²⁴⁴⁸ followed by nuclear translocation in RAEC. Similarly, hypoxia induced phosphorylation of the mTORC2-substrate Akt-Ser⁴⁷³ (3-6 h at 1% O₂) and a brief phosphorylation peak of the mTORC1-substrate S6K-Thr³⁸⁹ (10-60 min). Phosphorylation of Akt was inhibited by mTOR knockdown and partially with rapamycin. mTOR knockdown, rapamycin or Akt inhibition specifically and significantly inhibited proliferation of serum-starved RAEC under hypoxia (P<0.05; n ≥ 4). Similarly, hypoxia induced Akt-dependent and rapamycin-sensitive proliferation in mouse embryonic fibroblasts. This response was partially blunted by HIF-1α knockdown and not affected by TSC2 knockout. Finally, mTORC2-inhibition by rictor silencing especially (P<0.001; n=7), and mTORC1-inhibition by raptor-silencing partially (P<0.05; n=7), inhibited hypoxia-induced RAEC proliferation.

Thus, mTOR mediates an early response to hypoxia via mTORC1 followed by mTORC2 promoting endothelial proliferation mainly via Akt-signaling. mTORC1 and especially mTORC2 might therefore play important roles in diseases associated with hypoxia and altered angiogenesis.
INTRODUCTION

Hypoxia is associated with angina pectoris, myocardial infarction, heart failure and peripheral artery disease. Hypoxia and tissue ischemia is either caused by arterial obstruction or functional and anatomical capillary rarefaction because of hypertension\(^1\). Hypoxia occurs during rapid tissue growth, in organ and in tumor development and during chronic inflammation or exposure to high altitude\(^1\). Diminished oxygen concentration induces programmed responses such as endothelial proliferation\(^2,3\) and angiogenesis which ultimately relieves tissue hypoxia and contributes to wound healing\(^4\).

We have reported that hypoxia requires mammalian target of rapamycin (mTOR) to induce angiogenesis and cell proliferation of the vascular wall in response to hypoxia\(^5\). The mTOR pathway is a key regulator of cell growth and proliferation and increasing evidence suggests that its deregulation is associated with human diseases, including cancer, diabetes and cardiovascular disease\(^6\). The mTOR pathway integrates signals from nutrients, energy status and growth factors to regulate many processes, including autophagy, ribosome biogenesis and metabolism\(^6\). Recent work identified two structurally and functionally distinct mTOR-containing multiprotein complexes\(^7,8\): The first complex, mTORC1, harbors raptor, and is highly rapamycin-sensitive,\(^9-13\) and specifically activates protein synthesis via S6K. The second complex, mTORC2\(^13-15\), is associated with rictor and phosphorylates Akt on Ser\(^473\)\(^16,17\). mTORC2 phosphorylates and activates Akt/PKB, which promotes signaling pathways that ensure cell survival and induce cell proliferation\(^18\).
Reports on the effects of hypoxia on mTOR are contradicting; On the one hand, hypoxia activates mTOR-signaling to enhance angiogenesis \(^{19}\), cellular proliferation of lung adventitial fibroblasts \(^{20}\) and aortic wall cells \(^{5}\), or protein levels and activity of HIF-1\(\alpha\), a major transcription factor for hypoxia-inducible genes \(^{21}\). On the other hand, hypoxia has also been reported to inhibit mTOR signaling in mouse embryonic fibroblasts, which dephosphorylates S6K1 and downregulates protein synthesis \(^{22-24}\). It is unclear, how hypoxia can elicit both activation and inhibition of mTOR signaling and how these signals contribute to increased proliferation; Furthermore, it is currently not known, whether hypoxia affects mTORC2 and mTOR-dependent Akt-phosphorylation.

This study further assesses the effects of hypoxia on mTOR signaling in endothelial cells \(^{5}\). Here we examine activities of mTOR under hypoxia in detail and translation of this signal into endothelial cell proliferation.
MATERIALS AND METHODS

Rat aortic endothelial cells (RAEC) were prepared, cultured and characterized as described previously. Tsc2 defective MEFs were obtained from Michael Hall (Biocenter, Basel, Switzerland), HIF-1α knockouts from Max Gassman (University of Zürich, Switzerland). Endothelial cell spheroids were generated as described elsewhere. Predesigned siRNAs against rat mTOR (frap1_3 siRNA) were purchased from Qiagen. shRNA containing vectors against raptor an rictor are pKDM-132, a pSuper.gfp/neo-based 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 AMAXA nucleofection. Cell numbers were assessed using Cell Proliferation Reagent WST-1 (Roche Molecular Biochemicals) according to the manufacturer’s specifications.

Cell lysis was prepared as described previously and as described elsewhere. Primary chicken polyclonal anti-HIF-1α antibodies were provided by Max Gassmann (University of Zürich, Switzerland), polyclonal anti-raptor and anti-rictor antibodies were generated by Markus A. Rüegg (Biocenter Basel, Switzerland). All other antibodies are commercially available. Protein bands were analyzed by densitometric quantification by ImageJ 1.31v software (Wayne Rasband, NIH). Immunestaining was performed as described previously.

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 Graphpad software Prism. The number of single experiments compiled is indicated by n. A value of $P<0.05$ was considered as significant.

RESULTS

Hypoxia rapidly and concentration-dependently promotes phosphorylation of mTOR-Ser 2448 and mTOR nuclear translocation

To investigate direct effects of hypoxia on mTOR activity we have performed time-course experiments in serum-deprived cultured aortic endothelial cells, in the absence of growth factors. We determined phosphorylation of mTOR at Ser$^{2448}$ and Ser$^{2481}$ in the presence and absence of rapamycin. As shown in figure 1A, phosphorylation of Ser$^{2448}$ rapidly increases after exposure to hypoxia (1% O$_2$), peaks at around 3h of hypoxia and remains at high levels during the period investigated (24 h). The effect of hypoxia on Ser$^{2448}$ phosphorylation is reduced by rapamycin (Fig. 1A). In contrast, phosphorylation of mTOR Ser$^{2481}$ increased only slightly during hypoxic exposure and declined towards 24h of incubation. mTOR protein levels were not affected by hypoxia (Fig 1A).

In mammalian organs, O$_2$ concentration ranges from 14% to 0.5%, with 14% O$_2$ in arterial blood and 10% in the myocardium. During mild hypoxia, myocardial O$_2$ drops to 1% to 3% or lower. To account for varying oxygen concentrations in the body, we investigated the effect of different oxygen saturations on mTOR Ser$^{2448}$ phosphorylation. Quiescent RAEC were separately incubated under decreasing oxygen saturations (20%, 11%, 6%, 3% and 1% O$_2$) for 12 h. At normoxia (21% O$_2$), faint phosphorylation of mTOR Ser$^{2448}$ was detected, which increased when O$_2$
concentration was lowered to 11 – 6% and augmented further with a maximum at 1-
3% O2 (Fig.1B). mTOR protein- as well as β-actin protein levels were not affected by
oxygen saturation. HIF-1α protein levels were used as a positive control for hypoxia
and increased linearly peaking at 1-3% of O2 saturation (Fig. 1B). Thus, mTOR
phosphorylation on Ser2448 is modulated in the pathophysiologic O2 concentration
range.
An additional regulatory mechanism of mTOR signaling may occur via cytoplasmic-
nuclear shuttling 29. We examined whether severe hypoxia (1% O2) influences
cellular localization of mTOR and mTOR P-Ser2448. Under all tested conditions,
mTOR was localized predominantly in the cytosol as shown by immunostaining in
Fig. 1C. However, after quiescent RAEC were cultured in hypoxia for 6 h, mTOR
protein also appeared in the nucleus, and rapamycin treatment inhibited nuclear
localization (Fig. 1C). Interestingly, mTOR-P-Ser2448 was only detected in distinct
nuclear structures after 6 h of exposure to hypoxia. Phosphorylation of mTOR Ser2448
was not detected under normoxia and only a very faint signal was detected under
conditions of hypoxia with rapamycin treatment (Fig. 1C). Similar results were
obtained when assessing protein levels of mTOR and mTOR P-Ser2448 by Western
blotting. Serum-deprived RAEC were exposed to hypoxia (1% O2) for different
periods of time (2 – 24 h). At normoxia (Time point = 0) basal levels of HIF-1α were
detected in nuclear extracts, whereas the levels of mTOR and mTOR-P-Ser2448 were
nearly undetectable (Fig. 1D). mTOR and mTOR-P-Ser2448 protein levels appeared in
the endothelial nuclear fraction after 2 h of incubation under 1% O2, increased slightly
with time and were maximal after 24 h of incubation under 1% O2 (Fig. 1D). Thus,
hypoxia rapidly and dose-dependently promotes phosphorylation of mTOR-Ser\textsuperscript{2448} in a rapamycin-sensitive way and causes nuclear translocation of phosphorylated mTOR.

**Hypoxia induces rapid, but short-term mTOR–dependent phosphorylation of S6K1-Thr389 and sustained phosphorylation of Akt-Ser473**

mTOR is present in two complexes, mTORC1 and mTORC2. mTORC1 activity can be measured by analyzing the phosphorylation of the direct downstream target S6K1 on Thr\textsuperscript{389} or phosphorylation of ribosomal subunit S6\textsuperscript{30,31}. mTORC2 phosphorylates Akt on the primary phosphorylation site Ser\textsuperscript{473}\textsuperscript{16,17}. We therefore performed time course experiments, where quiescent RAEC were exposed to hypoxia (1% O\textsubscript{2}) for short (10 min) to long (24 h) term and we analyzed phosphorylation of S6K1-Thr\textsuperscript{389} and Akt-Ser\textsuperscript{374} phosphorylation. As shown representatively in figure 2A (first three panels) and as averaged densitometric quantification of cumulative experiments in Fig. 2B, upper graph, S6K1 was highly phosphorylated at Thr\textsuperscript{389} between 10 min to 1 h of hypoxic exposure but dropped to undetectable levels after more than 3 h of culture under hypoxia. This phosphorylation step is highly rapamycin-sensitive. Akt phosphorylation at Ser\textsuperscript{473} slightly increased after 10 min, but reached maximal levels after 3 h of hypoxic exposure before staying at a steady level for up to 24 h. Total Akt levels remained unchanged under hypoxia (Fig 2A, middle four panels). Phosphorylation of Akt at Ser\textsuperscript{473} peaked after 3 h of exposure to hypoxia as shown in Figure 2B, lower graph, representing the ratio of Akt-P-Ser473 to total Akt. Akt phosphorylation was partially inhibited by rapamycin, however, rapamycin’s effect
increased with longer incubation (averaged densitometric quantification of cumulative experiments in Fig. 2B, lower graph).

Akt phosphorylates Ser$^{21}$ in Glycogen synthase kinase-3 (GSK3) $\alpha$ and Ser$^{9}$ in GSK3$\beta$ and thereby inactivates GSK3 function$^{32,33}$. Furthermore Akt and GSK3 are implicated in the regulation of cell cycle regulators Cyclin D1 and p21$^{33}$. Similar to Akt phosphorylation, GSK3$\beta$ was phosphorylated after 60 min of exposure to hypoxia as shown by westernblots of nuclear extracts in Fig. 2A. Cyclin D1 protein gradually accumulated after 30 min of hypoxia in the nuclear fraction, whereas cell cycle inhibitor p21 protein levels decrease and totally disappear after 24 h of RAEC cultivation under hypoxia (Fig 2B).

To further examine the role of mTOR on Akt-P-Ser$^{473}$ under hypoxia, mTOR protein expression was silenced by mTOR specific siRNAs that were nucleo-fected into RAEC prior to starvation and exposure to hypoxia. Quiescent RAEC were then again exposed to hypoxia (1% O$_2$) for short (10 min) to long (24 h) term and Akt-Ser$^{374}$ phosphorylation was analyzed by Western blotting. mTOR silencing as shown by the Western blots in Figure 2C and averaged densitometric quantification of cumulative data in Fig. 2D effectively blunted hypoxia-induced Akt-Ser$^{374}$ phosphorylation.

Thus, hypoxia induces sustained phosphorylation of Akt-Ser$^{473}$, peaking at around 3-6 h of hypoxia that is blunted by rapamycin or mTOR silencing. On the other hand, rapamycin sensitive S6K1 phosphorylation on Thr$^{389}$ peaks between 10 min to 1 h of exposure to hypoxia, but quickly drops to undetectable levels with further culture under hypoxia.
Hypoxia-enhanced endothelial proliferation is mTORC1- and mTORC2-dependent

We compared our previous findings in rat aortic angiogenesis with an angiogenesis assay of endothelial spheroids and endothelial proliferation assays using RAEC at severe hypoxia (1% O₂). Endothelial sprout-formation under 1% O₂ was more than twice as high when compared to the response under 21% O₂ (Fig. 3A). Rapamycin selectively inhibited additional sprout-formation observed under 1% O₂ at a low concentration (2 nM) (Fig. 3A). We have shown previously that hypoxia-enhanced angiogenesis in vitro is mainly due to enhanced proliferation. A similar response was observed for RAEC proliferation under 1% O₂; Hypoxia alone increased RAEC proliferation when compared to diluent normoxic control to about 1.5 fold (Fig. 3B). Low concentrations of rapamycin (2 nM) inhibited proliferation specifically under hypoxia (Fig. 3B, upper graph). Akt inhibition by Akt IV inhibitor lowered overall proliferation at higher concentrations (Fig. 3B, lower graph). Akt inhibitor was used within concentrations where cytotoxicity was absent, as shown by cytotoxicity test performed in RAEC (see supplemental figure).

To further assess the role of mTOR in transducing hypoxia into endothelial proliferation, we analyzed endothelial (RAEC) proliferation and mTOR–associated signaling after mTOR silencing. mTOR protein was consistently knocked down or reduced (up to 95%) by mTOR siRNA, whereas control siRNA had no effect on mTOR protein as shown by Western blotting (Figure 2C). After silencing, quiescent endothelial cells were cultured for 30 h under 1% O₂ and 21% O₂ and proliferation was measured. mTOR silencing significantly (P<0.05, n=4) decreased increased
proliferation under hypoxia compared to transfection with control si-RNA, whereas mTOR silencing had no significant (P>0.05, n=4) effects on proliferation under normoxia when compared to proliferation in endothelial cells that were transfected with control si-RNA (Fig. 3C).

To assess whether a specific mTOR complex is responsible for transducing hypoxia into endothelial proliferation, we knocked down raptor, specific for mTORC1, or rictor, specific for mTORC2 by nucleofection of RAEC with vectors containing specific shRNAs. As shown in the upper part of Figure 3D, shRNA silencing effectively inhibited expression of raptor or rictor proteins as compared to negative control transfection. Rictor but not raptor silencing also clearly decreased phosphorylation of mTORC2 downstream substrate Akt-Ser\(^{473}\) after 6 h of incubation under hypoxia (Fig. 3D). At these timepoints, S6K1 phosphorylation at Thr\(^{389}\) is repressed (Fig. 3D). Importantly, inhibition of mTORC2 by rictor silencing effectively blunted hypoxia-induced endothelial proliferation with no effect on proliferation under normoxia (P<0.001, n=5). Also raptor-silencing decreased hypoxia-induced proliferation significantly (P<0.05, n=3), however, not to the extent of rictor silencing. Thus, both mTORC1 and especially mTORC2 silencing significantly reduces hypoxia-induced endothelial proliferation.

**Tsc2 knockout does not blunt hypoxia-induced proliferation in mouse embryonic fibroblasts**

In order to extend the validity of our data to other cell types that are commonly used in molecular biology research we have assessed MEF cells for their proliferative
response under hypoxia. The broad availability of transgene MEFs allows for rapid and easy determination of the role of a specific gene.

Tsc2 has been implicated to regulate proliferation under hypoxia in mouse embryonic fibroblasts (MEFs) \(^\text{24}\). Tsc1 and Tsc2 proteins form a physical and functional complex in vivo and inhibit mTOR. Tsc2 is inactivated by Akt-dependent phosphorylation or nutrient availability, which destabilizes Tsc2 and disrupts its interaction with Tsc1 \(^\text{34,35}\). We therefore investigated whether a disrupted Tsc1/Tsc2 complex in Tsc2-defective MEFs affects hypoxia-induced cell proliferation when compared to wildtype MEFs or Tsc2 mutated MEFs with a reintroduced Tsc2 wild-type gene. As shown in Fig. 4A, proliferation was clearly increased in TSC2\(^{-/-}\) MEFs (upper panel), both under normoxia and hypoxia. Hypoxia-induced proliferation was decreased by rapamycin and the Akt inhibitor as demonstrated for endothelial cells (Fig. 4A, upper panel).

Importantly, proliferation in TSC2 defective MEFs was enhanced under hypoxia to a ratio comparable to intact MEFs. No significant decrease (\(P>0.05, n=3\)) was observed when comparing the ratio of proliferation indices under hypoxia to that under normoxia (Fig. 4A, lower graphs). These results suggest, that loss of Tsc2 generally increases proliferation but does not regulate the specific activating effects of hypoxia on mTOR-mediated proliferation in MEFs.

**mTOR induces HIF-1\(\alpha\)-dependent and -independent ways to promote mouse embryonic fibroblast proliferation under hypoxia**

mTOR was shown to contribute to the stabilization of HIF-1\(\alpha\) protein in cells exposed to hypoxia and is thus a positive regulator of HIF-1-dependent gene transcription,
We therefore asked whether HIF-1α, downstream of mTOR, is pivotal for increased cell proliferation under hypoxia. For this purpose we measured proliferation of wild-type MEFs and those lacking the HIF-1α gene. As shown in figure 4B, hypoxia-induced proliferation was only partially, though significantly (P>0.05, n=5) inhibited in HIF-1α−/− cells (open squares) as compared to wild-type cells under hypoxia (open circles). Both wildtype (filled circles) and HIF-1α−/− cells (filled squares) did not increase proliferation under normoxia. Increased proliferation under hypoxia was rapamycin-sensitive both in HIF-1α−/− cells and wildtype MEFs. To further assess, whether mTOR requires HIF-1α to induce proliferation under hypoxia, we overexpressed mTOR in wildtype and HIF-1α−/− cells (Fig 4C, upper panel) and measured proliferation (Fig 4C, lower left panel). mTOR overexpression increased overall proliferation in all conditions to approximately the same levels when compared to corresponding mock-transfected cells; the ratios (Fig 4C, lower right panel) between proliferation under hypoxia and under normoxia was the same in wildtype and in HIF-1α knockout MEFs. These results suggest, that HIF-1α is a partial but not crucial effector of mTOR-dependent hypoxia-induced proliferation in MEFs.
DISCUSSION

In this report we have investigated the mechanisms responsible for hypoxia-induced proliferation of serum- and growth factor deprived endothelial cells and found that mTORC1 and mTORC2, i.e. the large multidomain kinase mTOR and its regulatory associated proteins raptor and rictor, mediate an early response to hypoxia promoting endothelial proliferation via Akt-signaling. Our results also clarify the apparent contradiction in the mTOR-field arising from earlier observations, that hypoxia activates mTOR signaling\(^\text{38}\) resulting in angiogenesis\(^\text{19}\), proliferation\(^\text{5,20}\) and HIF-1α stabilization\(^\text{21}\) and at the same time appears to inhibit mTOR signaling as seen by deactivation of mTORC1 substrate S6K1, 4E-BP1 and protein synthesis\(^\text{22-24}\); Our data suggest that both mTORC1 and mTORC2 participate in the response to hypoxia in a cooperative and timed program, that allows an early activation and late inhibition of mTORC1 and delayed and maintained activation of mTORC2.

We demonstrate that hypoxia (1% \(O_2\)) induces phosphorylation of mTORC2 downstream target Akt-Ser\(^{473}\) (3-6 h) and a short phosphorylation peak at mTORC1 substrate S6K-Thr\(^{389}\) (10-60 min). Thus, hypoxia activates mTOR, S6K1 and Akt in different ways. mTORC1-signaling appears to be activated only at a very early stage and is inhibited with prolonged (>3h) exposure to hypoxia. In contrast, mTORC2-signaling is maintained; Akt-Ser\(^{473}\)-phosphorylation increased under hypoxia at more than 3h and was sustained in 1% \(O_2\). Importantly, phosphorylation of Akt was partially inhibited by rapamycin and strongly by mTOR silencing. It has initially been reported that mTORC2, i.e., the rictor-mTOR complex, is rapamycin-insensitive\(^\text{14,15}\). However, later studies have shown, that prolonged rapamycin treatment inhibits
mTORC2 assembly and in consequence, Akt/PKB in certain cell types including endothelial cells (HUVECs, in particular) \(^{39}\).

In line with these phosphorylation studies, mTOR silencing, rapamycin and Akt inhibition all specifically and significantly inhibited proliferation of serum-starved RAEC under hypoxia, and rapamycin also decreased endothelial sprout-formation in endothelial spheroids under hypoxia alone. Finally, rictor knockdown, and therefore inhibition of mTORC2 signaling, clearly decreased hypoxia-induced phosphorylation on Akt Ser\(^{473}\) and totally blunted hypoxia-induced endothelial proliferation. On the other hand, raptor silencing, and therefore inhibition of mTORC1, did not affect Akt phosphorylation and partially though significantly reduced hypoxia-induced endothelial proliferation.

The differences of hypoxic activation of mTOR1 and mTORC2 hypothetically may involve distinct effects of hypoxia-induced, phosphorylation of mTOR at Ser\(^{2448}\): We show that hypoxia rapidly (10 min) and concentration-dependent promotes rapamycin-sensitive and sustained phosphorylation of mTOR-Ser\(^{2448}\) and mTOR nuclear translocation in RAEC. Phosphorylation of mTOR-Ser\(^{2448}\) was modulated in the physiologic oxygen saturation range (1-11% O\(_2\)) also covering moderate hypoxic conditions. This is consistent with other responses to hypoxia to prevent or delay the onset of more severe hypoxia \(^{40}\). However, the functional significance of the mTOR phosphorylation site in Ser\(^{2448}\) is still unknown. Phosphorylation of this site has been suggested to be part of a feedback mechanism regulating mTOR activity \(^{41}\). Though, it is still unclear, whether this feedback loop is positive or negative and whether it affects mTORC1 or mTORC2 to the same extent \(^{7,41}\). Further investigations
assessing whether hypoxia-induced nuclear mTOR-Ser\textsuperscript{2448} phosphorylation is associated with a specific mTOR-complex or -function will therefore be necessary. As shown by immuno-fluorescence mTOR-Ser\textsuperscript{2448} phosphorylation is localized to subnuclear macromolecular structures resembling promyelocytic leukaemia (PML)-nuclear bodies. These PML-bodies represent distinct yet dynamic intra-nuclear structures involved in apoptosis, proliferation and senescence, and also associate with nuclear phosphorylated Akt\textsuperscript{42}. Indeed, very recently, PML was shown to be a novel suppressor of mTOR and neoangiogenesis during ischemia\textsuperscript{19}.

Hypoxia also induces proliferation in lung adventitial-\textsuperscript{20}, cardiac-\textsuperscript{28} and mouse embryonic (MEF)-\textsuperscript{24} fibroblasts. In order to extend the validity of our data to other cell types, we have assessed how MEF cells increase proliferation under hypoxia. In MEFs, a loss of Tsc2 confers a growth advantage to hypoxic cells\textsuperscript{24}, suggesting that hypoxia inhibits mTOR via the tuberous sclerosis complex (TSC). TSC, consisting of Tsc1 and Tsc2, is the main upstream inhibitor of mTOR activity. The disruption of the complex by Tsc2 phosphorylation results in mTOR activation\textsuperscript{38}. Indeed, we confirm that disrupting the Tsc2 gene increases proliferation under hypoxia. However, the same advantage is present in wildtype MEFs or Tsc2-mutated MEFs with a reintroduced Tsc2-wildtype gene. Increased proliferation to hypoxia, however, was specifically decreased by rapamycin and Akt-inhibitor. Based on these experiments we conclude that mTOR mediates hypoxia-induced cell proliferation independent of regulation by TSC. An autonomous role of mTOR, in sensing and transducing oxygen saturation was suggested by recent work revealing that a redox-sensitive switch may contribute to the regulatory mechanism that controls the mTOR pathway.
Furthermore, oxidative capacity as displayed by mitochondrial activity was shown to regulate mTORC1 assembly. The proliferation studies in HIF-1α knockout MEFs suggest that HIF-1α is a partial downstream effector of mTOR-dependent proliferation under hypoxia. However, mTOR can promote hypoxia-induced proliferation also in the absence of HIF-1α expression of mTOR in HIF-1α knockout MEFs. Still, further studies will have to assess the role of HIF-1α in mTOR-dependent proliferation in endothelial cells, as well as the contribution of mTORC1 and mTORC2 complexes to HIF-1α stability but also to the activity of cell cycle regulators such as Cyclin D1 and p21.

In conclusion (see Fig. 5) hypoxia-induced proliferation in endothelial cells requires signaling from both mTOR complexes, mTORC1 and mTORC2. mTOR activation by hypoxia is monitored by an early and sustained rapamycin-sensitive phosphorylation and nuclear translocation of mTOR, specifically phosphorylated at Ser2448. Activation of mTORC2 is monitored by a sustained phosphorylation of AktSer473, which is decreased by mTOR and mTORC2 silencing and partially by prolonged rapamycin treatment. On the other hand, mTORC1 (rapamycin)-dependent S6K1 phosphorylation at early timepoints (<3h) is likely involved in the early events that lead to hypoxia-mediated endothelial proliferation, whereas at later time-points (>3h) mTORC1 signaling is repressed as seen by complete de-phosphorylation of S6K-Thr389. Blunting of hypoxia-induced endothelial proliferation by siRNA-mediated knockdown of raptor or rictor, monitors the importance of mTORC1 and especially TORC2, respectively. This indicates cooperating mechanisms between signals from
both mTOR complexes in the response to hypoxia in endothelial cells. Thus, mTORC1 and specifically mTORC2 may be interesting novel targets to regulate hypoxia-induced endothelial cell proliferation and angiogenesis for inhibition of tumor vascularization and potential induction of reparative angiogenesis during ischemic cardiovascular disease.
LEGENDS

Figure 1. Hypoxia (1% O₂) mediates rapid, dose-dependent, sustained phosphorylation of mTOR Ser²⁴⁴⁸ and translocation to the nucleus in RAEC.

A) Westernblot (upper panel) showing total cell lysates of serum-deprived rat aortic endothelial cells (RAEC) exposed to increasing duration of hypoxia (10 min to 24 h) and probed for phosphorylated Ser²⁴⁴⁸, phosphorylated Ser²⁴⁸¹ and total mTOR in the presence or absence of rapamycin (20 nM). Averaged densitometric quantification (lower panel) shows rapid, sustained and rapamycin-sensitive phosphorylation on Ser²⁴⁴⁸ relative to total mTOR protein that was statistically significant after 30 min of hypoxia (p<0.001, n=4). Data are given as mean±SEM.

B) Quiescent primary RAEC were cultured for 12 h under normoxia (21% O₂) and decreasing oxygen saturations (11%, 6%, 3%, 1% O₂). Total cell lysates were subjected to SDS-PAGE and protein levels of mTOR, mTOR Ser²⁴⁴⁸, HIF-1α and β-actin detected by immunoblotting.

C) Quiescent primary RAEC were cultured for 6 h under normoxia (21% O₂) (panel A), hypoxia (B) and hypoxia together with 20 nM rapamycin (C). Cells were fixed and immune-stained with anti-mTOR (FITC, green stain) and -mTOR phosphoserine 2448 antibodies (Cy3, red stain) and nuclear compartment (DAPI; blue stain). Rapamycin was included during serum deprivation (24 h).

D) Westernblot showing nuclear cell lysates of serum-deprived RAEC exposed to increasing periods of hypoxia (2 h to 24 h) and probed for phosphorylated Ser²⁴⁴⁸, HIF-1α and total mTOR.
Figure 2. Hypoxia increases transient or prolonged activity of distinct mTOR downstream targets

A) Western blots showing total cell lysates of serum-deprived RAEC exposed to increasing duration of hypoxia (10 min to 24 h) and probed for (numbering from top to bottom) (1) phosphorylated S6K-Thr$^{389}$, (2) phosphorylated S6K-Thr$^{389}$ in the presence of 20 nM rapamycin, (3) total S6K, (4) phosphorylated Akt-Ser$^{473}$, (5) total Akt, (6) phosphorylated Akt-Ser$^{473}$ and (7) total Akt in the presence of 20 nM rapamycin. Nuclear extracts probed for (8) phosphorylated GSK3-Ser$^{21/9}$ and (9) total GSK3, (10) Cyclin D1 and (11) p21.

B) Upper graph represent ratios of S6K P-Thr$^{389}$ to total S6K protein levels with and without rapamycin. Significant phosphorylation on S6K Thr$^{389}$ occurred within timepoints 10min to 180 min of hypoxia (P<0.05, n=3). Lower graph represents ratios of Akt P-Ser$^{473}$ to total Akt protein levels with and without rapamycin as calculated from compiled densitometric quantification. Akt Ser$^{473}$ phosphorylation was significant after 30 min of hypoxia (P<0.01, n=3). Rapamycin treatment resulted in significant reduction of phosphorylation after 180 min of hypoxia (P<0.05, n=3). Data are given as mean±SEM.

C) Western blots showing total cell lysates of serum-deprived RAEC transfected with negative control siRNA (+si-control) or siRNA directed against mTOR (+si-mTOR) exposed to increasing duration of hypoxia (10 min to 24 h) and probed for (numbering from top to bottom) (1) total mTOR, (2) phosphorylated mTOR-Ser$^{2448}$ (3) total Akt, (4) phosphorylated Akt-Ser$^{473}$. 
D) Graphs represent ratios of Akt P-Ser$^{473}$ to total Akt protein levels as calculated from compiled densitometric quantification. Phosphorylation on Akt Ser$^{473}$ was significant after 30 min of hypoxia (P<0.001, n=4). mTOR silencing significantly inhibited Akt P-Ser$^{473}$ after 30 min of hypoxia (P<0.05, n=4). Data are given as mean±SEM.
Figure 3. mTOR and downstream targets are required for hypoxia-induced endothelial cell proliferation

A) Typical micrographs (10x) of rat aortic endothelial spheroids (2000 cells) embedded in a fibrin gel after a 24 h incubation under normoxia (21% O₂) and hypoxia (1% O₂), normoxia (21% O₂) with 20 nM rapamycin and hypoxia (1% O₂) with 20 nM rapamycin. An additional panel shows a 40x magnification of endothelial sprouts emerging into the fibrin gel under hypoxia.

B) Cell numbers of serum deprived (for 30 h) RAEC were determined after 24 h culturing under normoxia (21% O₂) or hypoxia (1% O₂) with inclusion of the indicated concentrations of Akt IV Inhibitor and rapamycin. Y-axis represents the mean of cell number, compiled from three experiments with octuplicate samples. Data are given as mean±SD, n=3.

C) RAEC were nucleofected with control siRNA (si control, dark columns) and siRNA directed against rat mTOR (si mTOR, hatched columns). Quiescent cells were exposed to hypoxia for 30 hr and proliferation was measured. Significance was calculated by repeated ANOVA followed by pairwise comparison with the Bonferroni post test (ns; P>0.05, *; P<0.05, n=4).

D) RAEC were nucleofected with control siRNA (c and dark columns) and vectors containing shRNA directed against raptor (rap and light columns) or rictor (ric and striped columns). Western blots (upper display) show efficiency of silencing and effects on Akt Ser⁴⁷³- and S6K Thr³⁸⁹-phosphorylation after 6 h incubation under hypoxia. Quiescent cells were exposed to hypoxia for 30 hr and proliferation was measured (lower display). Significance in was calculated by repeated ANOVA
followed by pairwise comparison with the Bonferroni post test ( *, P<0.05, **; P<0.001, n=5).

**Figure 4. Hypoxia-induced proliferation in mouse embryonic fibroblasts via mTOR does not depend on TSC2 and only partially on HIF-1α.**

**A)** Cell numbers of serum-starved TSC2 knock out (TSC2 -/-) and wild type MEF (upper panel) were determined after 30 h incubation under normoxia and hypoxia. Rapamycin or Akt inhibitor was included 30 min before incubation under hypoxia (upper graphs). Lower graph shows proliferation of wildtype, TSC2 mutated MEFs with a reintroduced TSC2 wildtype gene (TSC2mt-wt-intro) and TSC2 mutated (TSC2mt) MEFs as ratio of proliferation under hypoxia divided by proliferation under normoxia (ns; P>0.05, n=3).

**B)** Cell numbers of serum-starved HIF-1α knock out (squares) and wild type MEF (circles) were determined after after 30 h incubation under normoxia (filled symbols) and hypoxia (open symbols). Rapamycin (2-200 nM) was included 30 min before incubation under hypoxia. Significance was calculated by one-way ANOVA followed by multiple comparison with the Bonferroni post test (***/ P<0.001, **; P<0.01, *; P<0.01, n=7).

**C)** HIF-1α knock out- and wild type MEFs were transfected with mock and HA-tagged wildtype mTOR and expression verified by westernblot (upper panel). Cell numbers of serum-starved mock-transfected- (dark columns) and mTOR-transfected (open columns) were determined after 30 h incubation under normoxia and hypoxia. The ratios between proliferation under normoxia and hypoxia were compared and no statistical difference among all groups was observed as calculated by one-way
ANOVA followed by multiple comparison with the Bonferroni post test (ns; P>0.05, n=3).

Figure 5. Scheme representing summary of most important results.
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.
ACKNOWLEDGMENTS

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CONFLICT(S) OF INTEREST

none
REFERENCES


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Figure 1B

mTOR P-Ser^{2448}
mTOR
HIF-1α
β-actin

1 3 6 11 21

O_2 (%)
Figure 1D

- **nuclear**
- **mTOR P-Ser^{2448}**
- **mTOR**
- **HIF-1α**

<table>
<thead>
<tr>
<th>Time (h) in 1%O_2</th>
</tr>
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<tbody>
<tr>
<td>0</td>
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Figure 2C

- mTOR
  - +si-control
  - +si-mTOR
- mTOR P-Ser^{2448}
  - +si-control
  - +si-mTOR
- Akt P-Ser^{473}
  - +si-control
  - +si-mTOR
- Akt
  - +si-control
  - +si-mTOR

Time (min) at 1% O_{2}
Figure 2D

Phospho-/total protein (arbit. units) vs. Time (min in 1% O₂)

- ■ control siRNA
- □ mTOR siRNA
Figure 4A

The graphs depict the effect of different concentrations of Rapamycin and Aktl on the ratio of proliferation in wildtype (wt) and Tsc2^{-/-} cells under 21% O$_2$ and 1% O$_2$. The ratio of proliferation is shown for each condition, with error bars indicating standard deviation. The graphs show a significant increase in proliferation under 1% O$_2$ compared to 21% O$_2$ for both wildtype and Tsc2^{-/-} cells.

Below, the ratio of proliferation for different genotypes is shown: wildtype, Tsc2mt-wt-intro, Tsc2^{-/-}, and Tsc2mt. The ratio for wildtype and Tsc2mt-wt-intro is similar, while Tsc2^{-/-} and Tsc2mt show a slight increase. The dashed line at 1 represents normoxia.
Figure 4B

Graph showing the effect of rapamycin on A450nm-A590nm with different conditions for wt and HIF-1α−/− cells under 21% and 1% O2. The data points are indicated with asterisks for statistical significance:

- *** <0.001
- ** <0.01
- * <0.05

Key:
- wt 21% O2
- wt 1% O2
- HIF-1α−/− 21% O2
- HIF-1α−/− 1% O2

X-axis: Rapamycin concentrations (0 nM, 2 nM, 20 nM, 200 nM)
Y-axis: A450nm-A590nm
Figure 4C

The figure shows a Western blot analysis of HA-tagged proteins under different oxygen conditions. The blot is stained with antibodies against HA and β-actin. The blot is probed with samples from mock and mTORwt conditions under 21% O₂ (mock, mTORwt) and 1% O₂ (mock, mTORwt).

Below the blot, a bar graph displays the quantification of the bands. The x-axis represents different conditions, and the y-axis shows the A450nm-A590nm absorbance values. The bars indicate the relative intensity of bands for both wild type (wt) and HIF-1α⁻/⁻ conditions.

Additionally, a second bar graph below shows the ratio of proliferation 1% O₂ vs 21% O₂ for wt and HIF-1α⁻/⁻ conditions, indicating no significant difference (ns).
Figure 5

Hypoxia

\[ \text{mTOR}^{\text{P-Ser}^{2448}} \]

\[ \text{Raptor} \leftrightarrow \text{mTORC1} \]

\[ \text{Rictor} \leftrightarrow \text{mTORC2} \]

\[ \text{P-Thr}^{389} \text{S6K1} \]

\[ \text{Akt}^{\text{P-Ser}^{473}} \]

Endothelial proliferation