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Akt1 regulates pathological angiogenesis, vascular maturation and permeability *in vivo*

Juhua Chen[1,4], **Payaningal R Somanath**[1,4], **Olga Razorenova**[1], **William S Chen**[2], **Nissim Hay**[2], **Paul Bornstein**[3], and **Tatiana V Byzova**[1]

1*Department of Molecular Cardiology, Joseph J. Jacobs Center for Thrombosis and Vascular Biology, NB50, The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA.*

2*Department of Biochemistry and Molecular Genetics, College of Medicine, University of Illinois at Chicago, 900 South Ashland Avenue, Chicago, Illinois 60607, USA.*

3*Department of Biochemistry, Box 357350, University of Washington, Seattle, Washington 98195, USA.*

Abstract

Akt kinases control essential cellular functions, including proliferation, apoptosis, metabolism and transcription, and have been proposed as promising targets for treatment of angiogenesis-dependent pathologies, such as cancer and ischemic injury. But their precise roles in neovascularization remain elusive. Here we show that Akt1 is the predominant isoform in vascular cells and describe the unexpected consequences of Akt1 knockout on vascular integrity and pathological angiogenesis. Angiogenic responses in three distinct *in vivo* models were enhanced in *Akt1*$^{-/-}$ mice; these enhanced responses were associated with impairment of blood vessel maturation and increased vascular permeability. Although impaired vascular maturation in *Akt1*$^{-/-}$ mice may be attributed to reduced activation of endothelial nitric oxide synthase (eNOS), the major phenotypic changes in vascular permeability and angiogenesis were linked to reduced expression of two endogenous vascular regulators, thrombospondins 1 (TSP-1) and 2 (TSP-2). Re-expression of TSP-1 and TSP-2 in mice transplanted with wild-type bone marrow corrected the angiogenic abnormalities in *Akt1*$^{-/-}$ mice. These findings establish a crucial role of an Akt-thrombospondin axis in angiogenesis.

The formation of a vasculature by vasculogenesis and angiogenesis is essential for embryonic development[1], tissue remodeling in adults and the unrestrained growth of tumors[2]. Vascular endothelial growth factor (VEGF) is widely accepted as a primary inducer of angiogenesis, but other factors have recently emerged as important regulators, including extracellular matrix proteins and their integrin receptors[3]. The angiogenic response to VEGF begins with vascular leakage and the deposition of a provisional matrix consisting of blood-derived components; the activation of proteases eventually degrades the extracellular matrix, releasing endothelial cells. The migration and proliferation of endothelium results in the formation of an initial capillary network, which becomes mature upon recruitment of mural cells[3]. Many of the biological activities ascribed to VEGF involve the activation of the phosphoinositide 3-kinase (PI3 kinase)-Akt signaling cascade. The inter-relationship between VEGF and the Akt pathway *in vivo* is complex, because PI3 kinase–Akt activation in turn mediates VEGF production in

Correspondence should be addressed to T.V.B. (byzovat@ccf.org)..
[4]These authors contributed equally to this work.

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tissues[4]. Akt is known to regulate short-term cellular processes, its activation occurs almost immediately upon stimulation by several growth factors and activated Akt can then phosphorylate multiple substrates. Notably, Akt controls long-term responses by regulating protein expression at the level of transcription and translation[5]. Thus, as a result of the complexity of the signaling network downstream of Akt, its role in pathophysiological processes remains elusive.

Recent studies conducted using transgenic and knockout animals have indicated that our understanding of Akt signaling mechanisms is far from complete. Although the phenotype of animals lacking single Akt isoforms is relatively mild[6], Akt1-Akt2 double-knockout mice die shortly after birth[7]. Although reports of vascular functions in Akt-deficient and transgenic mice are limited, it is clear that the role of Akt signaling, though not predictable, is of biological significance[8].

To delineate the role of the Akt pathway in the regulation of the formation and integrity of the vasculature, we used mice with a genetic disruption of the *Akt1* gene. Because of the numerous downstream targets of Akt and the possibility of distinct and opposing short-term and long-term consequences of Akt activation[9], an analysis of the vascular phenotype and its underlying mechanisms represents an experimental challenge. We therefore focused on the tissue responses to VEGF administered through multiple routes, excluding models that involve the induction of endogenous VEGF, to streamline our search for downstream effects of Akt.

RESULTS

Akt1 is the predominant isoform of Akt in endothelial cells

We investigated the expression of all three Akt isoforms in endothelial cells from the aorta, lung and Matrigel implants and found that Akt1 is the predominant isoform in these cells (Fig. 1a). In response to VEGF-A stimulation, active Akt (Ser473-phosphorylated Akt) levels in endothelial cells from $Akt1^{-/-}$ mice were less than 25% of those in endothelial cells from wild-type animals (Fig. 1b). This finding suggests a prominent role for Akt1 in VEGF-induced responses. Western blots for individual Akt isoforms show that the absence of Akt1 resulted in only minor changes in Akt2 and Akt3 expression in various tissues (**Supplementary Fig. 1** online). Moreover, in the absence of Akt1, total Akt activity, measured by phosphorylation of GSK-3β, was reduced by at least three- to fourfold in nonstimulated endothelial cells as well as in response to VEGF (Fig. 1c).

Akt1 in VEGF-induced angiogenic responses *in vitro* and *ex vivo*

In a frequently used *ex vivo* aortic ring assay, the average length and branch number of outgrown microvessels were reduced by at least 40% using rings from $Akt1^{-/-}$ animals compared to those from wild-type animals (Fig. 1d). In an independent approach, we corroborated these observations by quantifying the outgrowth of CD31-expressing endothelial cells using FACS analysis (Fig. 1e). Based on the results of thymidine-incorporation assays, wild-type endothelial cells showed a 3.2-fold increase in proliferation in response to serum. In contrast, $Akt1^{-/-}$ endothelial cells showed only a modest 1.4-fold increase (data not shown). Thus, the proliferation rate of $Akt1^{-/-}$ endothelial cells *in vitro* is reduced.

As cell migration is an essential step in angiogenic response, we assessed the effect of Akt1 deficiency on endothelial cell migration. VEGF exerted a pronounced effect on the migration of wild-type endothelial cells on various matrix proteins, whereas the migration of $Akt1^{-/-}$ endothelial cells in response to VEGF was significantly impaired (Fig. 1f,g and **Supplementary Fig. 1** online). We have previously shown that VEGF induces integrin activation in endothelial cells[10], which can be quantified as an increase in fibrinogen binding. In a soluble ligand–binding assay, $Akt1^{-/-}$ endothelial cells bound three times less fibrinogen

than their wild-type counterparts (**Supplementary Fig. 1** online). We observed no changes in the expression levels of major integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$) and VEGF receptors (data not shown). Impaired integrin function often results in decreased extracellular matrix assembly and deposition[11], which in turn might affect the integrity of the endothelial monolayer and its permeability, an essential part of the angiogenic response[12]. As anticipated, *Akt1*$^{-/-}$ endothelial cell monolayers were ~50% more leaky than their wild-type counterparts in the presence and in the absence of VEGF stimulation (Fig. 1h).

Enhanced pathological angiogenesis in *Akt1*$^{-/-}$ mice

To investigate the role of Akt1 in neovascularization *in vivo*, we injected mice subcutaneously with Matrigel with or without VEGF. We observed larger plugs with a threefold increase in hemoglobin content in the presence of VEGF in *Akt1*$^{-/-}$ mice compared to wild-type mice (Fig. 2a). Quantification of endothelial cells in Matrigel showed that VEGF-induced vascular proliferation in *Akt1*$^{-/-}$ mice was enhanced by 40% (Fig. 2b), suggesting that the permeability of *Akt1*$^{-/-}$ blood vessels contributed greatly to the angiogenic response.

Pathological angiogenesis induced by tumor implantation was assessed in wild-type and *Akt1*$^{-/-}$ mice. In these experiments, B16F10 melanoma cells were implanted subcutaneously. Tumors grown in *Akt1*$^{-/-}$ mice were characterized by a considerable increase in the number of blood vessels compared to tumors grown in wild-type mice (Fig. 2c). On the periphery of tumors formed in wild-type mice, a substantial vasculature was present, but it was markedly decreased in the center. In contrast, tumors grown in *Akt1*$^{-/-}$ mice had a relatively equal distribution of blood vessels and the lack of vasculature in the center was not apparent. Notably, tumors grown in *Akt1*$^{-/-}$ mice had a significantly higher vascular density (Fig. 2d) and vascular area compared to wild-type controls (Fig. 2e). No differences were found in the vascularity of normal skin in wild-type versus *Akt1*$^{-/-}$ mice (data not shown), suggesting that the enhanced tumor angiogenesis was not the result of preexisting differences in blood vessel density. Notably, in two-thirds of the tumors grown in wild-type animals, we observed a substantial necrotic core (15% of the total tumor area on average; Fig. 2f). But none of the tumors grown in *Akt1*$^{-/-}$ mice showed any necrosis, indicating that increased tumor vascularization provided a more efficient blood supply in tumors grown in *Akt1*$^{-/-}$ compared to wild-type mice.

Akt1-deficient vasculature is immature and leaky

The majority of tumor-induced blood vessels were positive for CD105, a marker of newly formed blood vessels (Fig. 2g). The density of 'young' blood vessels was 2.3-fold higher in tumors implanted in *Akt1*$^{-/-}$ versus wild-type mice (Fig. 2h). Blood vessel maturation measured by smooth muscle–cell actin (SMA) staining was considerably reduced in the absence of Akt1 (Fig. 3a). In wild-type mice, 17% of total CD31$^+$ blood vessels stained positively for SMA, whereas only 9.5% of blood vessels formed in *Akt1*$^{-/-}$ mice were mature blood vessels (Fig. 3b). Moreover, immunostaining for laminin and subsequent confocal microscopic analysis showed a reduction of at least 50% in the thickness of basement membrane of blood vessels formed in *Akt1*$^{-/-}$ mice compared to those formed in wild-type mice (Fig. 3c,d). Details of the ultrastructure of blood vessels formed in wild-type and *Akt1*$^{-/-}$ mice are presented in **Supplementary Figure 2** online. Based on western blot analysis, laminin content in blood vessels was significantly reduced in tumors grown in *Akt1*$^{-/-}$ mice ($P = 0.031$; **Supplementary Fig. 2** online). Thus, *Akt1*$^{-/-}$ blood vessels are characterized by a less developed basement membrane and deficient recruitment of mural cells, which in turn could result in the leaky phenotype of the *Akt1*$^{-/-}$ vasculature. Indeed, plasma leakage, measured as an area positive for plasma-derived fibrin (Fig. 3e), was almost doubled in tumors implanted in *Akt1*$^{-/-}$ mice (Fig. 3f).

We observed similar results in a second model of transient angiogenesis induced by adenovirus carrying the gene that encodes VEGF-A (Ad-VEGF-A)[13]. In these experiments, Ad-VEGF-A but not control Ad-GFP stimulated a considerable angiogenic response, which reached a maximum 7–10 d after adenovirus injection (data not shown). At this time point, Ad-VEGF-A–induced vascularization in *Akt1*$^{-/-}$ mice was higher than that in their wild-type counterparts (Fig. 3g) Furthermore, in this model of angiogenesis, the proportion of mature SMA-positive blood vessels was at least 50% less in *Akt1*$^{-/-}$ mice compared to wild-type counter-parts (Fig. 3h).

Next, we used angiogenic growth factors with different activities to stimulate vascular permeability. VEGF-D, a growth factor that does not stimulate vascular permeability[14], caused a ~2-fold increase in vascular area in wild-type mice but not in *Akt1*$^{-/-}$ mice (Fig. 4a). A truncated form of VEGF-D (*i.e.*, VEGF-D$^{\Delta N\Delta C}$), which is able to induce vascular permeability[14], stimulated increases in vascular area of 2.4- and 4.3-fold in wild-type and *Akt1*$^{-/-}$ mice, respectively (Fig. 4a). These results show that enhanced vascular leakage in *Akt1*$^{-/-}$ mice contributes greatly to increased angiogenic response.

As anticipated from previously published studies and from our own *in vitro* analysis, the basal proliferation rate of endothelial cells in the untreated skin of *Akt1*$^{-/-}$ mice is much lower than that in wild-type mice, as judged by staining for the proliferation marker Ki67 (Fig. 4b). But 7 d after treatment with Ad-VEGF-A, endothelial cell proliferation increased 2.5- and 15-fold in skin in wild-type and *Akt1*$^{-/-}$ animals, respectively. Thus, it seems that VEGF-A–stimulated proliferation of endothelial cells *in vivo* was not impaired by the lack of Akt1.

Notably, the permeability of preexisting blood vessels in response to a proinflammatory stimulus was 2.4-fold higher in *Akt1*$^{-/-}$ mice compared to wild-type mice (Fig. 4c). In addition, we observed increased deposition of a plasma-derived provisional matrix (which stained positively for fibrin) in *Akt1*$^{-/-}$ mice in response to Ad-VEGF-A but not to control Ad-GFP (Fig. 4d,e). This finding was corroborated by the results of ricin staining, which visualizes microvessel morphology and the sites of vascular leakage in whole mounts of ear skin[15]. Upon stimulation, we observed more intensive staining and increased density of leakage sites in the vasculature of *Akt1*$^{-/-}$ mice than in wild-type mice (Fig. 4f). Quantitative analysis showed increased areas of exposed basement membrane in vessels in *Akt1*$^{-/-}$ compared to wild-type mice (Fig. 4g). Thus, newly formed and preexisting blood vessels in the skin of *Akt1*$^{-/-}$ mice are defective and more permeable than those in wild-type mice. In our models, we observed no significant differences in the expression of angiopoietin-1 and angiopoietin-2, major regulators of vascular permeability, between wild-type and *Akt1*$^{-/-}$ mice (**Supplementary Fig. 2** online).

Akt1 deficiency results in changes of extracellular matrix

The observed changes in basement membrane matrix composition prompted us to perform an analysis of several other matrix proteins that are known to be involved in the regulation of angiogenesis. We observed a substantially reduced density of collagen fibrils and a reduced amount of total collagen in the skin of *Akt1*$^{-/-}$ compared to wild-type mice (**Supplementary Fig. 3** online). As a consequence, tumors grown in wild-type but not in *Akt1*$^{-/-}$ mice were densely packed and showed signs of solid (mechanical) stress and compression generated by interaction of the expanding tumor with the extracellular matrix (**Supplementary Fig. 3** online). Thus, the reduced stiffness of the surrounding extracellular matrix, together with increased vascular permeability, may facilitate the growth of microvessels in *Akt1*$^{-/-}$ mice.

Several features of *Akt1*$^{-/-}$ mice (abnormalities in collagen fibril organization, increased vascular permeability and enhanced angiogenesis) closely resemble the phenotype of mice deficient in the matricellular proteins thrombospondin 1 (TSP-1) and thrombospondin 2

(TSP-2)[16]. Accordingly, we performed an analysis of TSP levels. TSP-1 expression in implanted tumors was limited to stromal cells, and was not present in tumor cells (Fig. 5a). The TSP-1–positive area in tumors grown in *Akt1*$^{-/-}$ mice was significantly smaller than that in wild-type tumors (Fig. 5b). Notably, the tumor vascular density correlated inversely with the expression level of TSP-1 (Fig. 5c and **Supplementary Fig. 4** online), suggesting a role for TSP-1 in the regulation of tumor angiogenesis. Western blot analysis of tissue lysates showed reduced expression of both TSP-1 and TSP-2 in tumors grown in *Akt1*$^{-/-}$ mice as compared to those grown in wild-type mice, as well as reduced expression in the skin of *Akt1*$^{-/-}$ compared to wild-type mice (Fig. 5d). Endothelial cells isolated from *Akt1*$^{-/-}$ mice retained their *in vivo* characteristics and expressed reduced levels of TSP-1 and TSP-2 when compared to endothelial cells from wild-type mice (Fig. 5e and **Supplementary Fig. 4** online).

Akt1 controls expression of TSPs

Decreased levels of TSP-1 in *Akt1*$^{-/-}$ mice correlated with the extent of Akt activation (Fig. 5e). Upregulation of Akt activity in *Akt1*$^{-/-}$ endothelial cells by treatment with adenovirus encoding a constitutively active myristolated (myr) form of Akt1 (Ad-myrAkt1) resulted in a considerable increase in TSP-1 expression (Fig. 5e). Next, we transfected endothelial cells with a plasmid in which the *Thbs2* (encoding TSP-2) promoter (starting at nucleotide −2,701) controls expression of a luciferase reporter gene and cotransfected with dominant negative and constitutively active forms of Akt1. Inhibition of Akt1 activity by contransfection with retroviral constructs encoding a dominant negative Akt (DNAkt1) resulted in a nearly threefold decrease in luciferase levels (Fig. 5f). In contrast, the expression of myrAkt1 produced a modest but significant increase in luciferase levels over control, showing that Akt1 directly regulates expression of TSPs in endothelial cells.

Notably, exogenous TSP-1 was able to modulate the permeability of an endothelial cell monolayer (Fig. 5g). Moreover, downregulation of endogenous TSP-1 levels by siRNA in wild-type endothelial cells caused an increase in endothelial cell permeability (Fig. 5h), providing direct evidence that TSP-1 is a crucial factor in vascular permeability.

Role of an Akt1-TSP axis in angiogenesis

To confirm a direct role of Akt signaling in the regulation of endothelial cell permeability, we transfected *Akt1*$^{-/-}$ endothelial cells with Ad-myrAkt1 or an adenovirus carrying the gene encoding green fluorescent protein (GFP; Ad-GFP, control). Restoration of Akt1 activity with myrAkt1 in *Akt1*$^{-/-}$ endothelial cells reversed the leaky phenotype of these cells, whereas treatment with Ad-GFP had no effect (Fig. 6a). Notably, the hyperpermeability of blood vessels in *Akt1*$^{-/-}$ mice was rescued *in vivo* by prolonged treatment with Ad-myrAkt1 (Fig. 6b). Together, these data indicate that Akt1 signaling regulates vascular permeability and maturation, which in turn influence angiogenic responses *in vivo*.

In further analysis of the role that TSPs might have in abnormal angiogenesis in *Akt1*$^{-/-}$ mice, we considered that two major sources of TSPs could influence the course of an angiogenic response: TSP-1 released from platelets, and TSP-1 and TSP-2 expressed by endothelial and stromal cells[17]. Accordingly, to examine the contribution of platelets[18] and to assess the role of bone-marrow derived components in angiogenesis, we replaced the bone marrow of *Akt1*$^{-/-}$ mice with wild-type cells. Although this procedure diminished tumor angiogenesis and tumor cell survival somewhat in *Akt1*$^{-/-}$ mice, it did not result in a complete rescue of the phenotype. The vascular area in tumors quantified by CD31 staining, and tumor weight were decreased by 20% and 17%, respectively, in chimeras compared to *Akt1*$^{-/-}$ mice (data not shown). Thus, although bone marrow–derived cells are involved in the highly angiogenic phenotype of *Akt1*$^{-/-}$ mice, the local tissue environment seems to have a much more prominent role. In a further attempt to correct the phenotype of *Akt1*$^{-/-}$ mice, we restored TSP levels by

injecting cells expressing TSP-1 and TSP-2. Primary syngeneic endothelial cells were transfected with murine TSP-1 and TSP-2 and injected subcutaneously into chimeric *Akt1*$^{-/-}$ mice transplanted with wild-type bone marrow (**Supplementary Methods** online). Two weeks later, a mixture of melanoma cells and TSP-overexpressing cells were inoculated into the same areas, and tumors were collected after 1 week. Western blot analysis showed an increase in levels of both TSP-1 and TSP-2 in endothelial cells *in vitro*, as well as in tumors grown *in vivo* (Fig. 6c), and this result was further confirmed by immunostaining of tumor sections (Fig. 6d). As anticipated, increased TSP expression significantly suppressed tumor growth and tumor cell survival, as judged by tumor weight (Fig. 6e) and the area of necrosis *in vivo* (Fig. 6f). Notably, the density of blood vessels, as well as the proliferative rate of endothelial cells *in vivo*, was significantly reduced by TSP expression (Fig. 6d,g,h). Taking these data together, it seems that local re-expression of TSP-1 and TSP-2, combined with transplantation with wild-type bone marrow, is able to correct the phenotype of *Akt1*$^{-/-}$ mice.

Role of an Akt1-eNOS pathway in angiogenesis

Endothelial nitric oxide synthase (eNOS) is a known downstream target of Akt and might also contribute to the angiogenic phenotype of *Akt1*$^{-/-}$ mice, as described in a recent study[19]. Indeed, we observed a modest decrease in the basal levels of active eNOS in endothelial cells isolated from *Akt1*$^{-/-}$ mice when compared to wild-type mice. But the presence of compensatory mechanisms is evident, as we observed only a small difference upon stimulation with VEGF (**Supplementary Fig. 5** online). To examine the role of eNOS (encoded by the *Nos3* gene), we assessed tumor-induced angiogenesis in *Nos3*$^{-/-}$ mice (**Supplementary Figs. 5** and **6** online). Similar to *Akt1*$^{-/-}$ mice, tumor blood vessels in *Nos3*$^{-/-}$ mice were considerably less mature than in wild-type, with an apparent lack of pericyte recruitment as evidenced by tissue costaining for CD31 and SMA (**Supplementary Fig. 6** online). But, in contrast to *Akt1*$^{-/-}$ mice, the vascular area in tumors grown in *Nos3*$^{-/-}$ mice was similar to that in wild-type mice (**Supplementary Fig. 6** online). Notably, we did not observe any changes in the expression of TSPs in *Nos3*$^{-/-}$ mice (data not shown). Thus, the lack of pericyte recruitment and vascular maturation observed in newly formed blood vessels of *Akt1*$^{-/-}$ mice might result from a lack of eNOS activity in *Akt1*$^{-/-}$ endothelial cells (**Supplementary Figs. 5** and **6** online). But enhanced angiogenesis and tumor survival in our models seem to be regulated by the composition of the extracellular matrix.

DISCUSSION

The Akt pathway might regulate angiogenic responses by several distinct and possibly counterbalancing mechanisms. For example, Akt is known to mediate hypoxia-induced expression of VEGF *in vitro* and *in vivo*[20,21], but its long-term activation decreases VEGF expression and vascularity in cardiac tissues[9]. Here, we find that a number of endothelial cell responses involved in angiogenesis, including capillary formation *ex vivo*, endothelial cell proliferation and endothelial cell migration in response to VEGF *in vitro*, were impaired in *Akt1*$^{-/-}$ endothelial cells, but that the permeability of an endothelial monolayer was increased, which would be expected to have a proangiogenic effect. Although these data again point to the multiple and complex roles of Akt1 in endothelial cell responses induced by VEGF, they raise the question of which effects of Akt1 will determine the course of the angiogenic response *in vivo*. To address this question, we assessed three distinct *in vivo* models of angiogenesis—angiogenesis in Matrigel, in implanted tumors and upon stimulation with Ad-VEGF. Our results showed that the lack of Akt1 causes enhanced angiogenesis. Thus, we conclude that the overall effect of Akt1 on angiogenesis is suppressive. In contrast, a recent study[19] reported impaired angiogenesis in *Akt1*$^{-/-}$ mice in hindlimb ischemia as well as upon treatment with Ad-VEGF-A. Because of the transient nature of Ad-VEGF-A–induced angiogenesis[13], it is possible that initial neovascularization (5 d after treatment)[19] might be delayed in *Akt1*$^{-/-}$ as

compared to wild-type mice. In this study, we showed that at the peak of the angiogenic response, which occurs 7–10 d after Ad-VEGF-A treatment, *Akt1*$^{-/-}$ mice developed extensive vasculature. To reconcile these results, we propose that Akt1 might have different roles at different stages of angiogenesis. Moreover, the short-term and long-term consequences of Akt1 activation might be distinct. In support of this interpretation, another recent study showed that chronic but not short-term overexpression of Akt1 in cardiac tissues suppresses angiogenesis[9], a finding consistent with our results.

In seeking to define the mechanisms of the effects of Akt1 on angiogenesis, we found that neovasculature in *Akt1*$^{-/-}$ mice was defective in pericyte recruitment, resulting in reduced maturation and a significant decrease in laminin deposition and basement membrane thickness. This feature of blood vessels in *Akt1*$^{-/-}$ mice might be a result of the insufficient short-term activation of one of the downstream targets of Akt, eNOS, as a similar defect in maturation was observed in eNOS-deficient (*Nos3*$^{-/-}$) mice[22]. We observed deficient maturation and increased permeability of blood vessels in *Akt1*$^{-/-}$ mice in all models of angiogenesis studied here. The direct role of Akt1 in the maintenance of vascular permeability was further supported by our observation that prolonged but not short-term expression of Akt1 corrected excessive vascular permeability *in vitro* and *in vivo*.

The majority of angiogenic growth factors, including VEGF-A, initiate their action by stimulation of vascular permeability, resulting in the deposition of a 'provisional' matrix for endothelial cell migration and formation of endothelial tubes[23]. An increased amount of provisional matrix might promote endothelial cell migration and stimulate a more profound angiogenic response. Notably, only growth factors that induce vascular leakage were able to generate a highly angiogenic phenotype in *Akt1*$^{-/-}$ mice. It has also been reported that bone marrow-derived cells might contribute to angiogenesis[24]. The replacement of bone marrow of *Akt1*$^{-/-}$ mice with wild-type cells confirmed the role of bone marrow–derived components in our model, but we did not observe complete rescue.

Another factor that seems to contribute to the increased angiogenesis and increased tumor cell viability in *Akt1*$^{-/-}$ mice is a reduction in skin collagen content. High collagen density increases the mechanical resistance of the matrix, which in turn reduces tumor growth; conversely, a loose matrix permits the formation of large, well-vascularized tumors[25,26]. Abnormally loose extracellular matrix represents an easier target for proteases, which have an essential role in the regulation of angiogenesis[27]. The same consideration applies to the laminin-deficient basement membranes of blood vessels in *Akt1*$^{-/-}$ mice, which might permit the accelerated release and mobilization of endothelial cells from preexisting vascular structures.

At a mechanistic level, vascular leakage and enhanced angiogenesis in *Akt1*$^{-/-}$ mice are directly linked to abnormalities in extracellular matrix composition, which, in turn, are long-term consequences of deficient Akt1 activation, and, therefore, might not be observed in models that involve short-term modulation of the Akt1 pathway. The regulation of extracellular matrix content by Akt1 might provide an explanation for the recently reported cardiac dysfunction and hyper-trophy in mice that overexpress Akt1[9]. An essential component of this biologically important connection is an observed decrease in the amount of TSP-1 and TSP-2 in the tissues of *Akt1*$^{-/-}$ mice. Indeed, our results showed that Akt1 directly controls the expression of TSPs at the transcriptional level. Regulation of extracellular matrix seems to require a prolonged activation of Akt1 and is distinct from the role of this kinase as a signaling molecule that is quickly and transiently activated in response to growth factors. This new function of Akt1 is independent of eNOS activation, as we observed no changes in TSPs levels in *Nos3*$^{-/-}$ mice. TSPs are known to inhibit neovascularization by several mechanisms, including induction of endothelial cell apoptosis through interaction with CD36 (ref. 28), inhibition of metalloproteinase activity, which in turn might prevent mobilization of endothelial cells[29],

and inhibition of cell-cycle progression[30]. The reduced levels of TSPs at the sites of angiogenesis in *Akt1*$^{-/-}$ mice could account for a lack of endothelial cell apoptosis, a normal proliferation rate of endothelial cells *in vivo*, and increased angiogenesis in our *in vivo* models. Our finding that excessive angiogenesis was corrected by re-expression of TSP-1 and TSP-2 in the skin of *Akt1*$^{-/-}$ mice strongly supports a causal role for TSPs in this response.

In addition to their well-known effects on endothelial cell proliferation and apoptosis, our study emphasizes another notable function of TSPs, namely the maintenance of vascular integrity. The leaky vascular phenotype observed in TSP-2-deficient mice[31] also supports our conclusions. Finally, the reduced collagen content in *Akt1*$^{-/-}$ mice could be another consequence of the lack of TSPs, which are known to regulate collagen matrix assembly[32].

In our experimental models, impaired vascular maturation but not other aspects of vascular development seem to be related to the eNOS pathway in *Akt1*$^{-/-}$ mice. This finding is consistent with substantial evidence indicating that eNOS is not an exclusive target of Akt and, therefore, a complete overlap of phenotypes in *Akt1*$^{-/-}$ and *Nos3*$^{-/-}$ mice is not anticipated[33]. Indeed, recent studies using mice that overexpress Akt emphasize the role of other Akt effectors, such as mTOR, in the regulation of angiogenesis in cardiac tissues[9]. Thus, although eNOS undoubtedly represents an important component of the Akt signaling network in endothelial cells, other mechanisms contribute to both the pro- and antiangiogenic functions of Akt. It is also important to consider that different animal models and tissue environments might show different consequences of Akt signaling.

In summary, our study shows that Akt1 is vital for the regulation of vascular permeability, angiogenic responses and subsequent vascular maturation. Our results and recent reports[9,34,35] suggest that Akt activity requires fine-tuning in a time-dependent manner. It is also clear that because of its numerous downstream targets, Akt1 affects several regulatory pathways of the vascularization response, including pro- as well as antiangiogenic components. In our models using *Akt1*$^{-/-}$ mice, changes in extracellular matrix and reduction in the levels of TSP-1 and TSP-2, endogenous inhibitors of angiogenesis, shifted the balance toward increased angiogenesis. As neoangiogenesis and vascular leakage are important pathophysiological features of cancer, ischemic injury, inflammation and degenerative disorders, our findings have broad therapeutic implications.

METHODS

Animals

We generated *Akt1*$^{-/-}$ mice as previously described[6] and maintained them in the 129 R1/C57BL/6 background. We used sex-matched 8–12-week-old wild-type and *Akt1*$^{-/-}$ littermate mice for the study. We performed all procedures according to protocols approved by Cleveland Clinic Foundation Institutional Animal Care and Use Committee.

Aortic ring assay and isolation of endothelial cells

We performed aortic ring assays as previously described[36]. We stimulated vessel sprouting using 30 ng/ml of VEGF for 14 d. We harvested endothelial cells outgrown from rings and quantified them by FACS analysis using CD31-specific antibody. We used the same protocol for the isolation of endothelial cells. We isolated microvascular endothelial cells from lungs as previously described[37]. For isolation of endothelial cells from Matrigel implants, we used a similar protocol.

Western blot analysis

We lysed tissue and tumor samples and performed SDS-PAGE and western blotting as described previously[18].

Analysis of endothelial cells *in vitro*

We performed cell migration assays as previously described[38,39] using Transwell plates. We incubated endothelial cells from wild-type or *Akt1*$^{-/-}$ mice with or without VEGF for 4 h at 37 °C. We microscopically quantified migration (magnification, ×200). We performed analyses of fibrinogen binding, integrin and TSP expression by FACS as previously described[40].

Matrigel plug assay

We performed Matrigel plug assay as previously described[36]. On day 5, we removed Matrigel plugs and digested them using 5 ml Drabkin reagent (Sigma) and quantified neovessel formation using a hemoglobin assay according to the manufacturer's protocol. In a parallel experiment, we used the harvested Matrigel implants to isolate endothelial cells.

Tumor angiogenesis

We performed tumor angiogenesis as previously described[36] using B16F10 melanoma cells, and collected the tumors 7 d after injection as indicated. We processed tissue sections as previously described[41].

Expression and analyses of Ad-VEGF-A_{165},Ad-VEGF-$D^{\Delta N\Delta C}$ and Ad-VEGF-D *in vivo*

We stimulated angiogenesis in skin using adenoviruses carrying genes encoding VEGF-A_{165}, VEGF-$D^{\Delta N\Delta C}$, VEGF-D or green fluorescence protein (GFP) as negative control as previously described[13]. Alternatively, we injected recombinant VEGF-A_{165}, VEGF-D and VEGF-$D^{\Delta N\Delta C}$ subcutaneously along with 0.1 ml of Matrigel. We isolated skin along with the muscle layer 7 d after injection and processed samples for immunohistochemistry. The means of von Willebrand factor (vWF)-positive vascular area were 1.5% and 1.9% for wild-type and *Akt1*$^{-/-}$ control samples, respectively.

Bone marrow transplantation

We lethally irradiated both wild-type and *Akt1*$^{-/-}$ 6–8-week-old recipient mice (n = 11 per group), and we transplanted 1×10^7 bone marrow cells from wild-type littermates by tail vein injection as previously described[42].

Immunohistochemistry and image analysis

We analyzed vessel density and area as described previously[13], based on vWF, CD31 and laminin staining. We detected the expression of TSP-1, vWF, CD105, fibrin and SMA using antibodies from Abcam, Dako, BD Biosciences, Accurate Chemical and Scientific Corporation and Sigma, respectively, in tissue sections by immunohistochemistry (**Supplementary Methods** online). We quantified the area of TSP-1 expression and plasma leakage using Image Pro-Plus software as previously described[39].

Vascular permeability assay *in vivo*

We performed this assay using Evans blue dye (30 mg/kg in 100 μl PBS; Sigma) as previously described[43] with modifications (**Supplementary Methods** online). We performed a characterization of microvessel morphology and leakage sites in ricin lectin-stained whole mounts of ear skin using a previously described protocol[15] with modifications (**Supplementary Methods** online).

Vascular permeability assay *in vitro*

We performed a vascular cell mono-layer permeability assay of primary endothelial cells isolated from wild-type and *Akt1*$^{-/-}$ animals as described in[44] with modifications (**Supplementary Methods** online).

Thbs2 promoter activity assay

We transiently transfected human umbilical vein endothelial cells (HUVECs) with a vector encoding luciferase under the control of the *Thbs2* promoter (starting at nucleotide −2,701) as described[45] or cotransfected them with retroviral constructs encoding dominant negative Akt1 (Akt1K179M) or myrAkt1 with vehicle as control. We determined luciferase activity in cell lysates with a luciferase assay kit (Promega) using a Wallac Victor^2 1420 Multilabel counter (Perkin Elmer) and normalized the result to total protein as measured by a protein assay kit (Bio-Rad).

Statistical analysis

All data are presented as means ± s.e.m. for *in vivo* assays or means ± s.d. for *ex vivo* studies and were analyzed using SPSS software (Norusis SPSS Inc., Release 6.0). We performed all analyses using two-sample t-tests with a two-tailed significance set at the 0.05 level. Before any test, we examined normality of the data and performed appropriate transformation when the normal distribution assumption was not met. We reported the actual *P* value for each test. In addition, we examined the relationship between TSP-1 expression intensity and vessel density using Pearson correlation coefficient with a significance level of 0.01.

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References

1. Risau W. Mechanisms of angiogenesis. Nature 1997;386:671–674. [PubMed: 9109485]
2. Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. Nat. Med 1995;1:27–31. [PubMed: 7584949]
3. Carmeliet P. Angiogenesis in health and disease. Nat. Med 2003;9:653–660. [PubMed: 12778163]
4. Jiang BH, Zheng JZ, Aoki M, Vogt PK. Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. Proc. Natl. Acad. Sci. USA 2000;97:1749–1753. [PubMed: 10677529]
5. Kandel ES, Hay N. The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. Exp. Cell Res 1999;253:210–229. [PubMed: 10579924]
6. Chen WS, et al. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev 2001;15:2203–2208. [PubMed: 11544177]
7. Peng XD, et al. Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. Genes Dev 2003;17:1352–1365. [PubMed: 12782654]
8. O'neill BT, Abel ED. Akt1 in the cardiovascular system: friend or foe? J. Clin. Invest 2005;115:2059–2064. [PubMed: 16075047]
9. Shiojima I, et al. Disruption of coordinated cardiac hypertrophy and angiogenesis contributes to the transition to heart failure. J. Clin. Invest 2005;115:2108–2118. [PubMed: 16075055]
10. Byzova TV, et al. A mechanism for modulation of cellular responses to VEGF: activation of the integrins. Mol. Cell 2000;6:851–860. [PubMed: 11090623]

11. Ruoslahti E, Engvall E. Integrins and vascular extracellular matrix assembly. J. Clin. Invest 1997;99:1149–1152. [PubMed: 9077520]
12. Carmeliet P, et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat. Med 2001;7:575–583. [PubMed: 11329059]
13. Byzova TV, et al. Adenovirus encoding vascular endothelial growth factor-D induces tissue-specific vascular patterns *in vivo*. Blood 2002;99:4434–4442. [PubMed: 12036873]
14. Tammela T, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. Cardiovasc. Res 2005;65:550–563. [PubMed: 15664381]
15. Thurston G, et al. Leakage-resistant blood vessels in mice transgenically overexpres-sing angiopoietin-1. Science 1999;286:2511–2514. [PubMed: 10617467]
16. Agah A, Kyriakides TR, Lawler J, Bornstein P. The lack of thrombospondin-1 (TSP1) dictates the course of wound healing in double-TSP1/TSP2-null mice. Am. J. Pathol 2002;161:831–839. [PubMed: 12213711]
17. Bornstein P. Thrombospondins as matricellular modulators of cell function. J. Clin. Invest 2001;107:929–934. [PubMed: 11306593]
18. Chen J, et al. Impaired platelet responses to thrombin and collagen in AKT-1 deficient mice. Blood 2004;104:1703–1710. [PubMed: 15105289]
19. Ackah E, et al. Akt1/protein kinase Balpha is critical for ischemic and VEGF-mediated angiogenesis. J. Clin. Invest 2005;115:2119–2127. [PubMed: 16075056]
20. Mazure NM, Chen EY, Laderoute KR, Giaccia AJ. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-ras-transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood 1997;90:3322–3331. [PubMed: 9345014]
21. Arsham AM, Plas DR, Thompson CB, Simon MC. Akt and hypoxia-inducible factor-1 independently enhance tumor growth and angiogenesis. Cancer Res 2004;64:3500–3507. [PubMed: 15150104]
22. Yu J, et al. Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. Proc. Natl. Acad. Sci. USA 2005;102:10999–11004. [PubMed: 16043715]
23. van Hinsbergh VW, Collen A, Koolwijk P. Role of fibrin matrix in angiogenesis. Ann. NY Acad. Sci 2001;936:426–437. [PubMed: 11460496]
24. Aicher A, et al. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. Nat. Med 2003;9:1370–1376. [PubMed: 14556003]
25. Hotary KB, et al. Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix. Cell 2003;114:33–45. [PubMed: 12859896]
26. Puolakkainen PA, Brekken RA, Muneer S, Sage EH. Enhanced growth of pancreatic tumors in SPARC-null mice is associated with decreased deposition of extracellular matrix and reduced tumor cell apoptosis. Mol. Cancer Res 2004;2:215–224. [PubMed: 15140943]
27. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ. Res 2003;92:827–839. [PubMed: 12730128]
28. Simantov R, Silverstein RL. CD36: a critical anti-angiogenic receptor. Front. Biosci 2003;8:s874–s882. [PubMed: 12957861]
29. Rodriguez-Manzaneque JC, et al. Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. Proc. Natl. Acad. Sci. USA 2001;98:12485–12490. [PubMed: 11606713]
30. Armstrong LC, et al. Thrombospondin 2 inhibits microvascular endothelial cell proliferation by a caspase-independent mechanism. Mol. Biol. Cell 2002;13:1893–1905. [PubMed: 12058057]
31. Lange-Asschenfeldt B, et al. Increased and prolonged inflammation and angiogenesis in delayed-type hypersensitivity reactions elicited in the skin of thrombospondin-2–deficient mice. Blood 2002;99:538–545. [PubMed: 11781236]
32. Bornstein P, Agah A, Kyriakides TR. The role of thrombospondins 1 and 2 in the regulation of cell-matrix interactions, collagen fibril formation, and the response to injury. Int. J. Biochem. Cell Biol 2004;36:1115–1125. [PubMed: 15094126]

33. Shiojima I, Walsh K. Role of Akt signaling in vascular homeostasis and angiogenesis. Circ. Res 2002;90:1243–1250. [PubMed: 12089061]
34. Sun JF, et al. Microvascular patterning is controlled by fine-tuning the Akt signal. Proc. Natl. Acad. Sci. USA 2005;102:128–133. [PubMed: 15611473]
35. Nagoshi T, et al. PI3K rescues the detrimental effects of chronic Akt activation in the heart during ischemia/reperfusion injury. J. Clin. Invest 2005;115:2128–2138. [PubMed: 16007268]
36. Reynolds LE, et al. Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat. Med 2002;8:27–34. [PubMed: 11786903]
37. Dong QG, et al. A general strategy for isolation of endothelial cells from murine tissues. Characterization of two endothelial cell lines from the murine lung and subcutaneous sponge implants. Arterioscler. Thromb. Vasc. Biol 1997;17:1599–1604. [PubMed: 9301641]
38. Byzova TV, Kim W, Midura RJ, Plow EF. Activation of integrin alpha(V)beta(3) regulates cell adhesion and migration to bone sialoprotein. Exp. Cell Res 2000;254:299–308. [PubMed: 10640428]
39. De S, et al. Molecular pathway for cancer metastasis to bone. J. Biol. Chem 2003;278:39044–39050. [PubMed: 12885781]
40. Narizhneva NV, et al. Thrombospondin-1 up-regulates expression of cell adhesion molecules and promotes monocyte binding to endothelium. FASEB J 2005;19:1158–1160. [PubMed: 15833768]
41. De S, et al. VEGF-integrin interplay controls tumor growth and vascularization. Proc. Natl. Acad. Sci. USA 2005;102:7589–7594. [PubMed: 15897451]
42. Takahashi T, et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat. Med 1999;5:434–438. [PubMed: 10202935]
43. Han ED, MacFarlane RC, Mulligan AN, Scafidi J, Davis AE III. Increased vascular permeability in C1 inhibitor-deficient mice mediated by the bradykinin type 2 receptor. J. Clin. Invest 2002;109:1057–1063. [PubMed: 11956243]
44. Stockton RA, Schaefer E, Schwartz MA. p21-activated kinase regulates endothelial permeability through modulation of contractility. J. Biol. Chem 2004;279:46621–46630. [PubMed: 15333633]
45. Soula-Rothhut M, et al. The tumor suppressor PTEN inhibits EGF-induced TSP-1 and TIMP-1 expression in FTC-133 thyroid carcinoma cells. Exp. Cell Res 2005;304:187–201. [PubMed: 15707585]

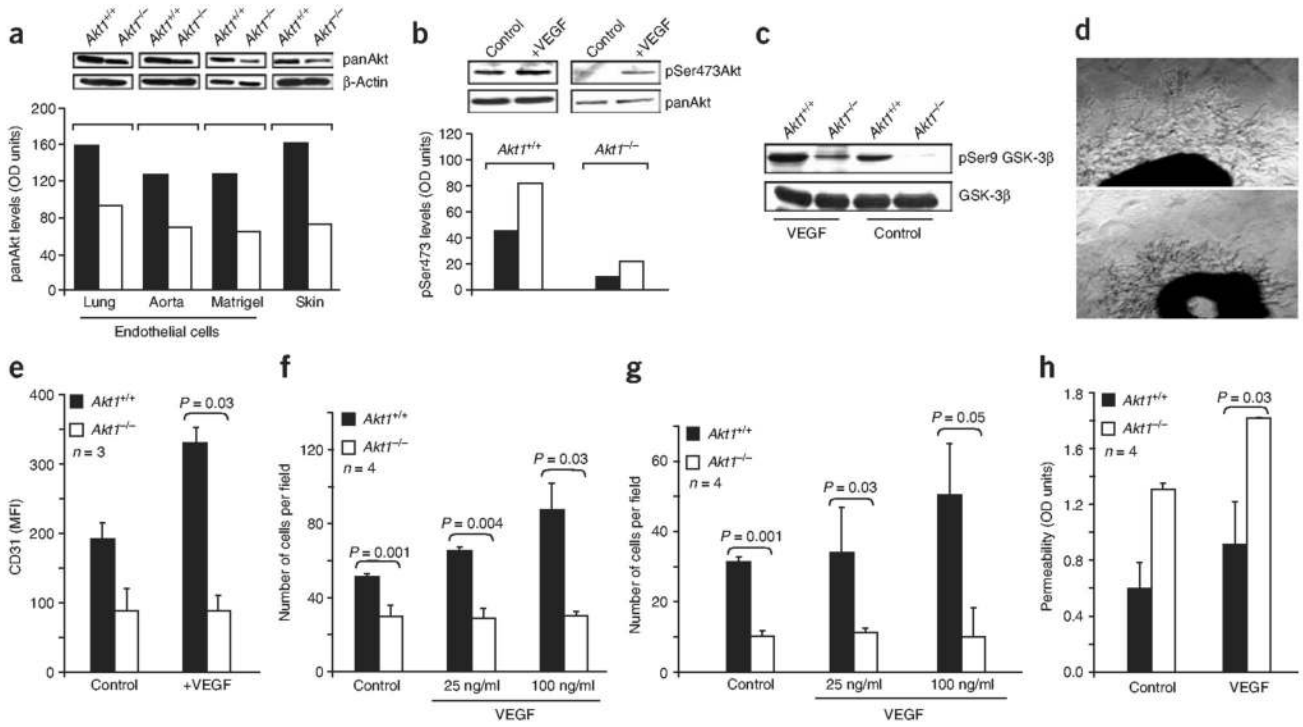

Figure 1.

Deficiency of Akt1, the predominant Akt isoform in endothelial cells, impairs their function *ex vivo*. (**a**) Western blot analyses and band densitometry of total Akt expression (using panAkt antibodies) in endothelial cells isolated from lungs, aorta and Matrigel plugs and in skin of wild-type (filled bars) and $Akt1^{-/-}$ (open bars) mice. (**b**) Western blot analyses of total phosphorylated Akt in lung endothelial cells from wild-type and $Akt1^{-/-}$ mice in response to 20 ng/ml VEGF. (**c**) Western blot analyses of phosphorylated GSK-3β in lung endothelial cells from wild-type and $Akt1^{-/-}$ mice in the presence and absence of VEGF (20 ng/ml). (**d**) Micrographs of representative aortic ring microvessels from wild-type (top) and $Akt1^{-/-}$ (bottom) animals grown in the presence of VEGF. Scale bars, 20 μm. (**e**) Quantification of vascular outgrowth in the presence or absence of VEGF (30 ng/ml) was performed by FACS analysis using CD31-specific antibodies. MFI, mean of fluorescence intensity. (**f,g**) Endothelial cell migration toward vitronectin (**f**) and fibronectin (**g**) was stimulated by 25 and 100 ng/ml of VEGF as indicated. (**h**) Permeability of endothelial monolayers *in vitro*. Wild-type and $Akt1^{-/-}$ endothelial cell monolayers were grown in transwells for at least 1 week to reach confluency and then stimulated with VEGF (30 ng/ml) or were unstimulated. *n* refers to number of times each experiment was repeated for each condition.

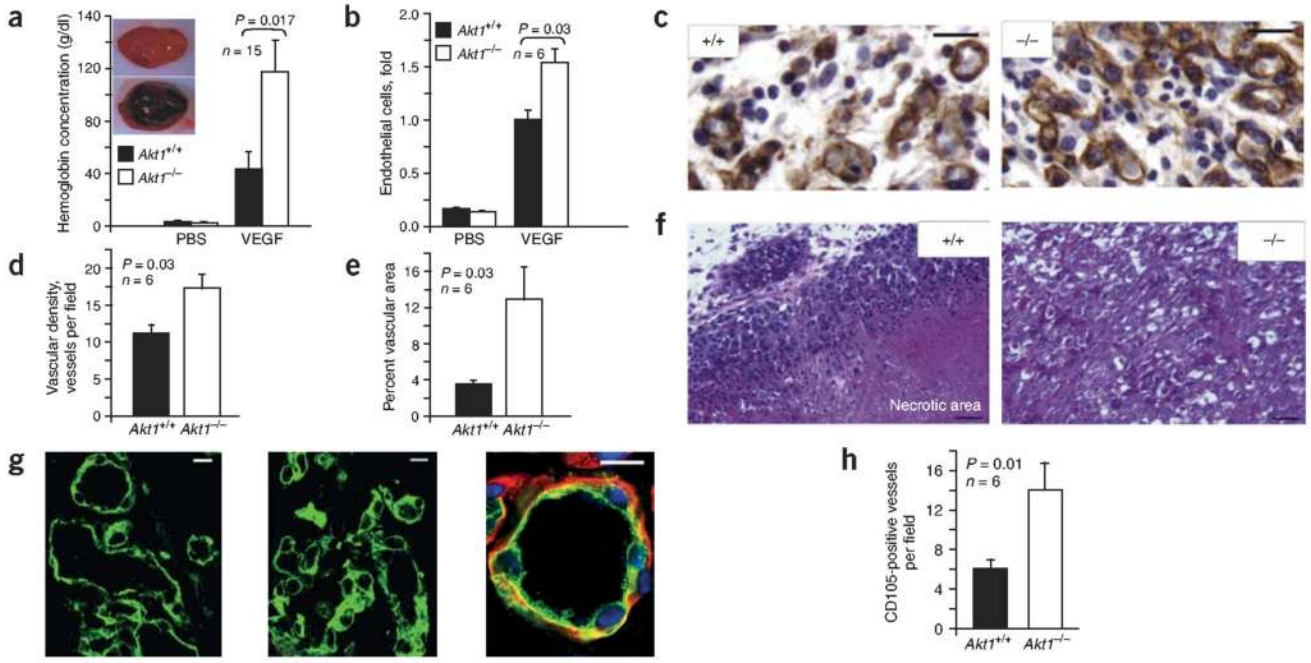

Figure 2.

Enhanced *in vivo* angiogenesis in *Akt1*$^{-/-}$ mice. (**a,b**) Angiogenesis in Matrigel implants stimulated with VEGF. (**a**) Hemoglobin content in Matrigel implants from *Akt1*$^{-/-}$ mice was higher compared to that from wild-type mice. Inset shows the microscopic appearance of 5-d-old Matrigel implants with 60 ng/ml VEGF from wild-type (top) and *Akt1*$^{-/-}$ (bottom) mice (original magnification, ×2). (**b**) Increased endothelial cell infiltration into Matrigel in *Akt1*$^{-/-}$ versus wild-type mice. (**c**–**e**) Angiogenesis induced by implanted tumors. (**c**) Comparison of CD31 staining of tumors grown in wild-type (left) and *Akt1*$^{-/-}$ mice (right). Scale bars, 20 μm. (**d**) Image analysis shows the increased density of blood vessels (the number of vessels per tissue area) in tumors grown in *Akt1*$^{-/-}$ versus wild-type mice. (**e**) Comparison of vascular area in tumors implanted in wild-type and *Akt1*$^{-/-}$ mice. (**f**) A representative photograph of the necrotic area in the center of tumor grown in wild-type mice (left). No necrosis was observed in tumors from *Akt1*$^{-/-}$ mice (right). Sections were stained with hematoxylin and eosin. Scale bars, 50 μm. (**g**) Increased number of CD105-positive blood vessels (green fluorescence) in frozen sections of tumors grown in *Akt1*$^{-/-}$ mice (middle) compared to wild-type mice (left). Right panel shows costaining for CD105 (green) and laminin (red) in the tumor blood vessels in wild-type mice. Nuclei are stained with DAPI. Scale bars, 10 μm. (**h**) Quantitative analysis of CD105-positive vessels in tumors from *Akt1*$^{-/-}$ mice compared to wild-type mice. *n* refers to the number of mice in each group.

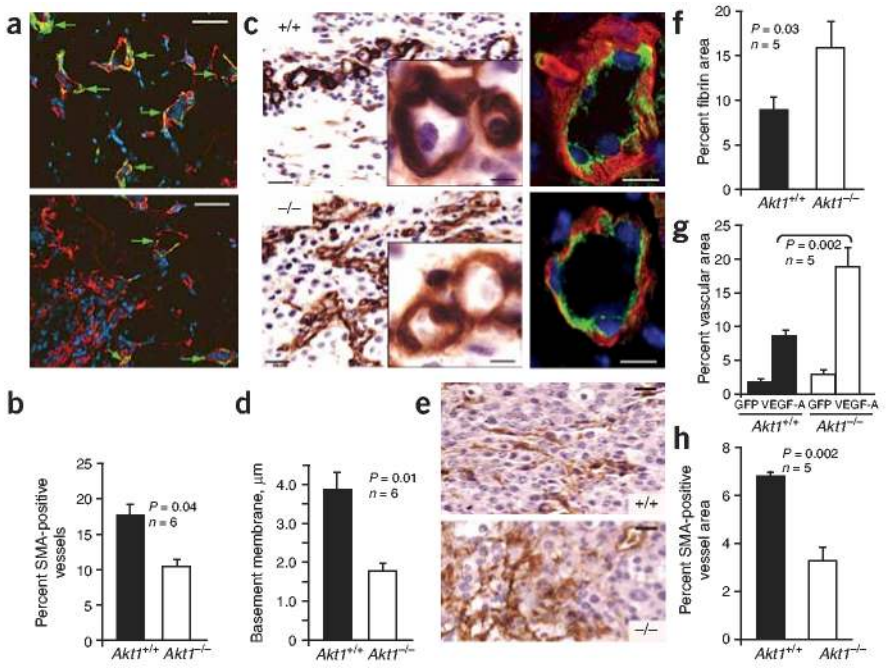

Figure 3.

Neovasculature in *Akt1*$^{-/-}$ mice is immature and leaky. Immunohistochemistry and image analysis of tumors implanted into wild-type and *Akt1*$^{-/-}$ mice. (**a**) Costaining for SMA (green) and CD31 (red) in tumors from wild-type and *Akt1*$^{-/-}$ mice. Nuclei are stained with DAPI. Arrowheads indicate SMA-positive vascular structures. Scale bars, 50 μm. (**b**) Quantification of the data presented in **a**. (**c**) Laminin-stained blood vessels in tumors grown in wild-type (top) and *Akt1*$^{-/-}$ mice (bottom). Insets show higher-magnification images. Scale bars, 20 μm. Inset scale bar, 5 μm. Immunohistochemistry (right panels) shows laminin (red), CD105 (green) and nuclei (blue). Scale bars, 10 μm. (**d**) Thickness of laminin-positive basement membrane in microvessels formed in wild-type and in *Akt1*$^{-/-}$ mice. (**e**) Fibrin deposition (brown) in tumors grown in wild-type (top) and *Akt1*$^{-/-}$ (bottom) mice. Scale bars, 20 μm. (**f**) Quantification of fibrin-positive area shown in **e**. (**g,h**) Angiogenesis induced by VEGF-A$_{165}$ or GFP (control) adenoviruses. (**g**) Comparison of vascular area in vWF-stained sections of wild-type and *Akt1*$^{-/-}$ skin upon stimulation with VEGF-A$_{165}$. Representative images are in **Supplementary Figure 2** online. (**h**) Comparison of SMA-positive vascular area in wild-type and *Akt1*$^{-/-}$ skin 7 d after injection of Ad-VEGF-A. Representative images are in **Supplementary Fig. 2** online.

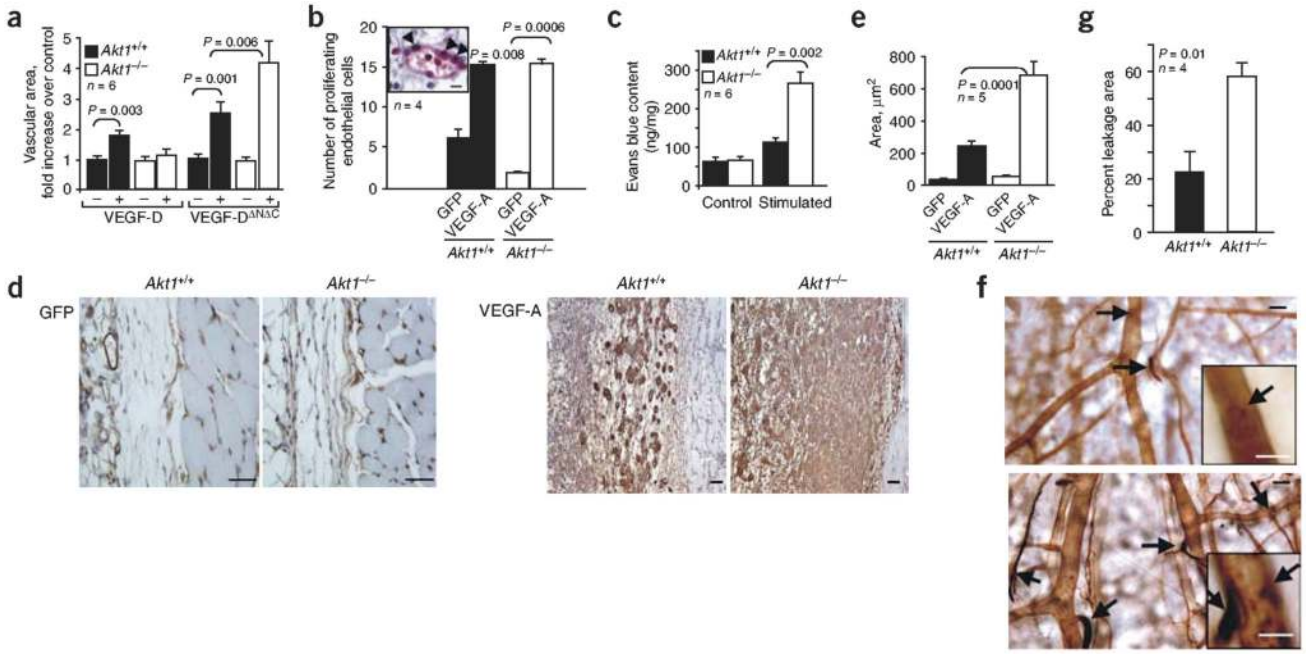

Figure 4.

Characterization of vascular responses in *Akt1*$^{-/-}$ mice. (**a**) Comparison of angiogenesis induced by full-length VEGF-D and VEGF-D$^{\Delta N\Delta C}$ in wild-type and *Akt1*$^{-/-}$ mice. (**b**) Comparison of endothelial cell proliferation in Ad-GFP– or Ad-VEGF-A–treated skin of wild-type and *Akt1*$^{-/-}$ mice based on Ki-67 staining. Inset shows a representative image of proliferating endothelial cells (arrowheads) in a skin blood vessel. Scale bar, 10 μm. (**c**) Plasma leakage in ear skin assessed by Evans blue extravasation after treatment with mustard oil, with no difference in control (unstimulated) ears. (**d**) Vascular permeability in wild-type and *Akt1*$^{-/-}$ mice in response to Ad-VEGF-A treatment. Fibrin staining shows increased leakage of fibrin in *Akt1*$^{-/-}$ compared to wild-type mice. Scale bars, 50 μm. (**e**) Area of fibrin deposition in skins from wild-type and *Akt1*$^{-/-}$ mice shown in **d**. (**f**) Comparison of ear microvessel morphology and leakage sites in ricin-stained whole mounts of ear skin in wild-type and *Akt1*$^{-/-}$ mice upon stimulation with mustard oil. Dark patches (shown by arrows) indicate sites of exposed basement membrane. There was an increased number of dark patches in blood vessels in *Akt1*$^{-/-}$ (lower panel, inset) compared to wild-type mice (upper panel, inset). Scale bars, 50 μm. Inset scale bar, 20 μm. (**g**) Quantitative analysis of the leakage area of wild-type and *Akt1*$^{-/-}$ blood vessels shown in **f**. *n* refers to the number of mice in each group.

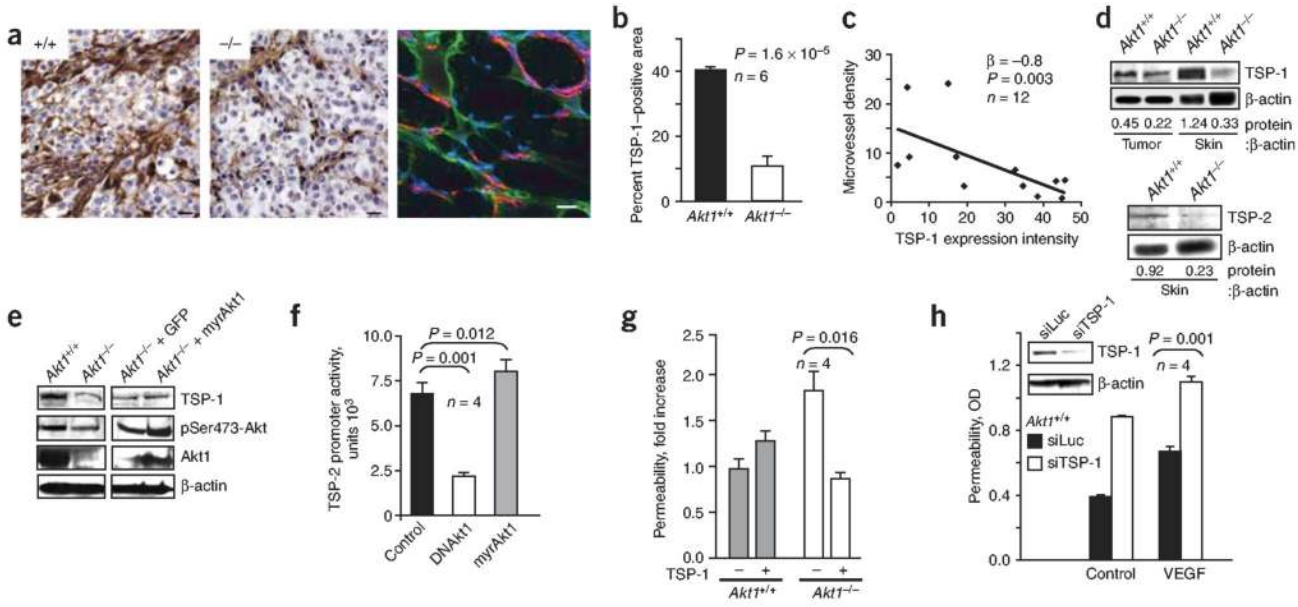

Figure 5.

Akt1 deficiency results in reduction of TSP levels. (**a**) TSP-1 staining in tumors from wild-type (left) and *Akt1*$^{-/-}$ mice (middle). Right panel shows costaining for TSP-1 (green) and CD31 (red) in tumors from *Akt1*$^{-/-}$ mice. Scale bar, 20 μm. (**b**) Comparison of TSP-1 staining in tumors from wild-type and *Akt1*$^{-/-}$ mice. (**c**) A reciprocal correlation existed between vascularity and TSP-1 expression in tumors. (**d**) Western blot and subsequent densitometry shows reduced levels of TSP-1 and TSP-2 in tissues from *Akt1*$^{-/-}$compared to wild-type mice. (**e**) Levels of TSP-1, phosphorylated Akt, Akt1 and β-actin in wild-type and *Akt1*$^{-/-}$ endothelial cells upon treatment with Ad-myrAkt1 or Ad-GFP determined by western blot. There were increased levels of TSP-1 upon re-expression of Akt1. (**f**) Luciferase activity measured in HUVECs cotransfected with a *Tsp 2* promoter/luciferase construct and retroviral constructs encoding DNAKt1, myrAkt1 or control. (**g**) Permeability of endothelial cell monolayers from wild-type and *Akt1*$^{-/-}$ mice in the presence or absence of TSP-1. Exogenous TSP-1 (1 μg/ml) decreased the permeability of *Akt1*$^{-/-}$ endothelial cell monolayers, whereas it slightly increased the permeability of wild-type endothelial cell monolayers. (**h**) The consequences of downregulation of TSP-1 by siRNA on the permeability of endothelial cell monolayers. Endothelial cells were treated with TSP-1 siRNA lentivirus (siTSP-1) or luciferase siRNA (siLuc) as a control. Inset shows TSP-1 levels after siRNA treatment. The permeability of endothelial cells was stimulated by 30 ng/ml VEGF-A_{165} as indicated. *n* refers to number of times each experiment was repeated for each condition.

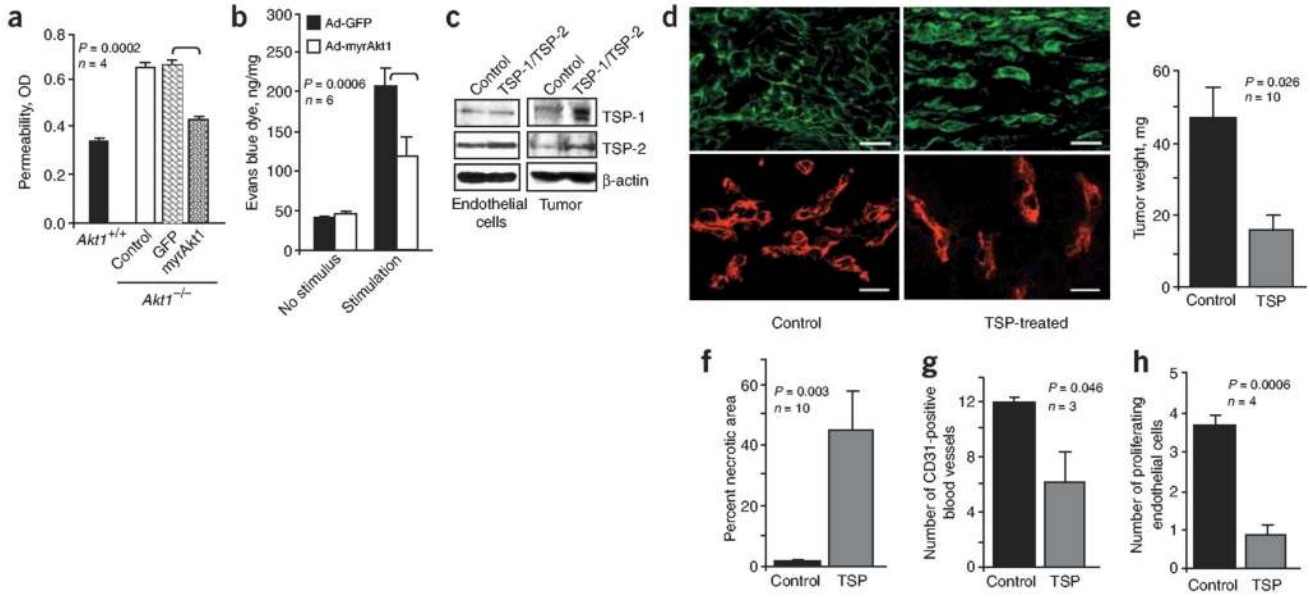

Figure 6.

An Akt1-TSP axis regulates vascular permeability and angiogenesis. (a) Comparison of the endothelial cell permeability of wild-type and *Akt1*$^{-/-}$ endothelial cells after treatment with Ad-myrAkt1 or Ad-GFP. Restoration of Akt resulted in the decrease of endothelial cell permeability to the level observed in wild-type endothelial cells. (b) Effect of prolonged Ad-myrAkt1 treatment on vascular permeability in *Akt1*$^{-/-}$ mice. Vascular leakage of Evans blue was stimulated by mustard oil (n = 6 mice per group). (c–h) TSP re-expression corrects the angiogenic phenotype of *Akt1*$^{-/-}$ mice. Primary endothelial cells and fibroblasts transfected with TSP-1 and TSP-2 were subcutaneously injected into *Akt1*$^{-/-}$ chimeric mice reconstituted with wild-type bone marrow followed by implantation of a mixture of tumor cells and TSP-overexpressing cells (**Supplementary Methods** online). (c) Western blot analysis shows increased expression of TSP-1 and TSP-2 *in vitro* in primary endothelial cells and fibroblasts cotransfected with plasmids carrying genes encoding TSP-1 and TSP-2. Increased TSP-1 and TSP-2 expression was also seen in the tumors grown *in vivo*. (d) Increased levels of TSP-1 (green staining, upper panels) and a decreased number of CD31-stained blood vessels (red, lower panels) in tumors upon re-expression of TSP-1 and TSP-2. Scale bar, 20 μm. (e) Effect of TSP-1 and TSP-2 on tumor weight. (f) Area of necrosis in tumors upon re-expression of TSP-1 and TSP-2. (g) Vascular density upon expression of TSP-1 and TSP-2 in tumors shown in d. (h) Expression of TSP-1 and TSP-2 resulted in decreased endothelial cell proliferation *in vivo* based on Ki67 staining (Fig. 4).