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IKKβ Suppression of TSC1 Links Inflammation and Tumor Angiogenesis via the mTOR Pathway

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SUMMARY

TNFα has recently emerged as a regulator linking inflammation to cancer pathogenesis, but the detailed cellular and molecular mechanisms underlying this link remain to be elucidated. The tuberous sclerosis 1 (TSC1)/TSC2 tumor suppressor complex serves as a repressor of the mTOR pathway, and disruption of TSC1/TSC2 complex function may contribute to tumorigenesis. Here we show that IKKβ, a major downstream kinase in the TNFα signaling pathway, physically interacts with and phosphorylates TSC1 at Ser487 and Ser511, resulting in suppression of TSC1. The IKKβ-mediated TSC1 suppression activates the mTOR pathway, enhances angiogenesis, and results in tumor development. We further find that expression of activated IKKβ is associated with TSC1 Ser511 phosphorylation and VEGF production in multiple tumor types and correlates with poor clinical outcome of breast cancer patients. Our findings identify a pathway that is critical for inflammation-mediated tumor angiogenesis and may provide a target for clinical intervention in human cancer.

INTRODUCTION

The microenvironment around tumors comprises several distinct types of cells, including fibroblasts, endothelial cells, and inflammatory cells. The identification of inflammatory cells in human tumors, first reported by Rudolf Virchow in 1863, led to the concept, now widely accepted, that the inflammatory cells in the tumor microenvironment play a critical role in promoting tumor progression, invasion, and angiogenesis (Balkwill and Mantovani, 2001). Among the factors secreted by tumor-associated-inflammatory cells, TNFα is a primary proinflammatory cytokine and is considered to be a potential mediator involved in several human diseases, including cancers. Recently, the TNFα/IKKβ signaling pathway has been suggested to link inflammation to cancer pathogenesis. It has been speculated that evasion of apoptosis (Greten et al., 2004) and insensitivity to antigrowth signals (Hu et al., 2004) contribute to TNFα-mediated tumor progression; however, these characteristics are not sufficient for cancer development—other factors, e.g., sustained angiogenesis, are also required (Hanahan and Weinberg, 2000). Although accumulating evidence suggests that angiogenesis plays a role in inflammation-mediated tumorigenesis, the underlying mechanism remains to be further investigated.

Tuberous sclerosis (TSC) is an autosomal-dominant disease characterized by tumors, which can grow in any organ of the body, and by numerous abnormal blood vessels around tumors. Two tumor suppressor genes, *TSC1* and *TSC2*, are associated with the development of TSC, and mutations in either gene are responsible for both the familial and sporadic forms of TSC (Young and Povey, 1998). TSC1 stabilizes TSC2 through binding with it, thereby preventing TSC2 from ubiquitination and degradation (Benvenuto et al., 2000). TSC2 acts as a GTPase-activating protein to regulate RHEB function through

converting RHEB from an active GTP-bound form to an inactive GDP-bound form (Garami et al., 2003; Inoki et al., 2003). GTP-bound RHEB is known to activate mTOR, whereas GDP-bound RHEB loses its ability to activate mTOR, suggesting that the TSC1/TSC2 complex can downregulate the mTOR pathway through suppressing RHEB function.

Upregulation of the TSC/mTOR signaling pathway has been implicated in the development of cancers. Activation of S6K1 and inactivation of 4EBP1 through phosphorylation by mTOR lead to increased protein synthesis, cell proliferation, and VEGF secretion and ultimately to tumorigenesis (Inoki et al., 2005). Activation of mTOR causes upregulation of VEGF through increases of both transcription (Brugarolas et al., 2003) and translation (Chung et al., 2002; Klos et al., 2006). Indeed, both *Tsc1* null and *Tsc2* null MEFs have increased VEGF production, an effect that is dependent on mTOR activation (El-Hashemite et al., 2003). In addition, patients with TSC develop renal acute myelogenous leukemia and skin angiofibromas that are characterized by numerous abnormal blood vessels (Young and Povey, 1998), and *Tsc1*- and *Tsc2*-heterozygous mice develop liver hemangiomas associated with extensive abnormal vasculization (Kwiatkowski et al., 2002; Onda et al., 1999), suggesting that the TSC/mTOR signaling pathway plays a role in regulating angiogenesis.

In this study, we attempted to determine whether angiogenesis plays a role in TNFα-mediated tumorigenesis. Unexpectedly, we discovered a previously unrecognized role of IKKβ: IKKβ upregulates mTOR activity through inactivation of TSC1. We concluded that dysregulation of the TSC/mTOR signaling pathway by IKKβ significantly contributes to inflammation-mediated cancer pathogenesis.

RESULTS

TNFα Activates the mTOR Pathway

TNFα is able to induce mTOR activation through the AKT pathway in certain cell types (Ozes et al., 2001). In an attempt to understand the molecular mechanism for TNFα-induced mTOR activation by examining the phosphorylation status of S6K1 at T389 [pS6K1(T389)] and 4EBP1 at S65 [p4EBP1(S65)], two well-known mTOR phosphorylation sites, we unexpectedly found that TNFα substantially induced pS6K1(T389) and p4EBP1(S65) within 5-10 min but caused virtually no activation of AKT within 20 min in breast cancer cell lines MCF-7 and MDA-MB-453 (Figure 1A). In vitro kinase assays further supported that mTOR and S6K1, but not AKT, were activated by TNFα stimulation (Figure 1B and Figure S1A in the Supplemental Data available with this article online). Since ERK and p38 also induce mTOR activation through AKT-independent pathways in human fibroblasts (Li et al., 2003; Ma et al., 2005), we examined whether ERK and p38 play roles in TNFα-induced mTOR activation. There was no significant ERK or p38 activation by TNFα within 30 min (Figures S1B and S1C) and depletion of AKT1/2/3, ERK1/2, and p38 by siRNA did not block TNFα-induced mTOR activation (Figures S2A, S2B, and S2C). Furthermore, inhibition of TNFα-induced pS6K1(T389) by mTOR siRNA supported the notion that TNFα-induced pS6K1(T389) requires mTOR (Figure 1C). Taken together, these results suggested that there may be an unknown signaling cascade—distinct from the AKT, ERK, and p38-mediated signaling pathways—by which TNFα activates mTOR in human epithelial breast cancers. Because activation of the mTOR signaling pathway by TNFα occurred within 10 min, it was unlikely that mTOR activation was due to transcriptional effects of NF-κB. As expected, suppression of nuclear translocation of NF-κB by the dominant-negative phosphorylation mutant IκBαM did not block TNFα-induced mTOR activation (Figure 1D), and transfection of NF-κB p65 did not upregulate mTOR activity (Figure 1E). In summary, these results suggested that TNFα stimulates mTOR signaling by a novel mechanism.

IKKβ Activates the mTOR Pathway

Since IKKβ is the major downstream kinase in the TNFα signaling pathway, we asked whether IKKβ plays a role in TNFα-induced mTOR activation. The pS6K1(T389) levels in multiple MCF-7 and MDA-MB-453 IKKβ stable transfectants were higher than those in the vector control or kinase-dead IKKβ mutant (nIKKβ) stable transfectants (Figure 2A). Similar results were obtained with transient expression (Figure 2B). This regulation was present not only in cancer cells but also in mammary epithelial cells derived from normal tissues (MCF-10A and HBL-100; Figures 2C and 2D), suggesting that TNFα- and IKKβ-induced mTOR activation is a general phenomenon. Furthermore, the pS6K1(T389) levels were lower in *Ikkβ*-deficient MEFs than in wild-type MEFs but were restored after IKKβ, but not IKKα, was added back (Figure 2E, left panel). Consistently, only IKKβ, but not IKKα, increased pS6K1(T389) levels in *Ikkα*-deficient MEFs (Figure 2E, right panel). siRNA-mediated knockdown of IKKβ caused a decrease in pS6K1(T389) (Figure 2F) and abrogated TNFα-induced pS6K1(T389) (Figure 2G, left panel). Since IKKγ plays an essential role in IKKβ activation, we examined whether IKKγ is required for TNFα-mediated mTOR activation. Indeed, siRNA-mediated knockdown of IKKγ significantly inhibited TNFα-induced mTOR activation (Figure 2G, right panel). The mTOR inhibitor rapamycin completely diminished pS6K1(T389) induced by TNFα and IKKβ but had much less effect on the level of pS6K1(T389) induced by a rapamycin-insensitive mTOR (S2035T) (Brown et al., 1995) (Figures 2H and 2I). Transfection of nIKKβ and pretreatment with two structurally unrelated IKKβ inhibitors—BAY 11-7082 and parthenolide—abrogated TNFα-induced pS6K1(T389) (Figures 2H and 2J), further supporting the crucial role of IKKβ in TNFα-induced mTOR activation.

Since the proinflammatory cytokine IL-1β and LPS function as IKKβ activators, we also examined whether mTOR activity is upregulated in response to IL-1β and LPS. As expected, IL-1β- and LPS-induced mTOR activations

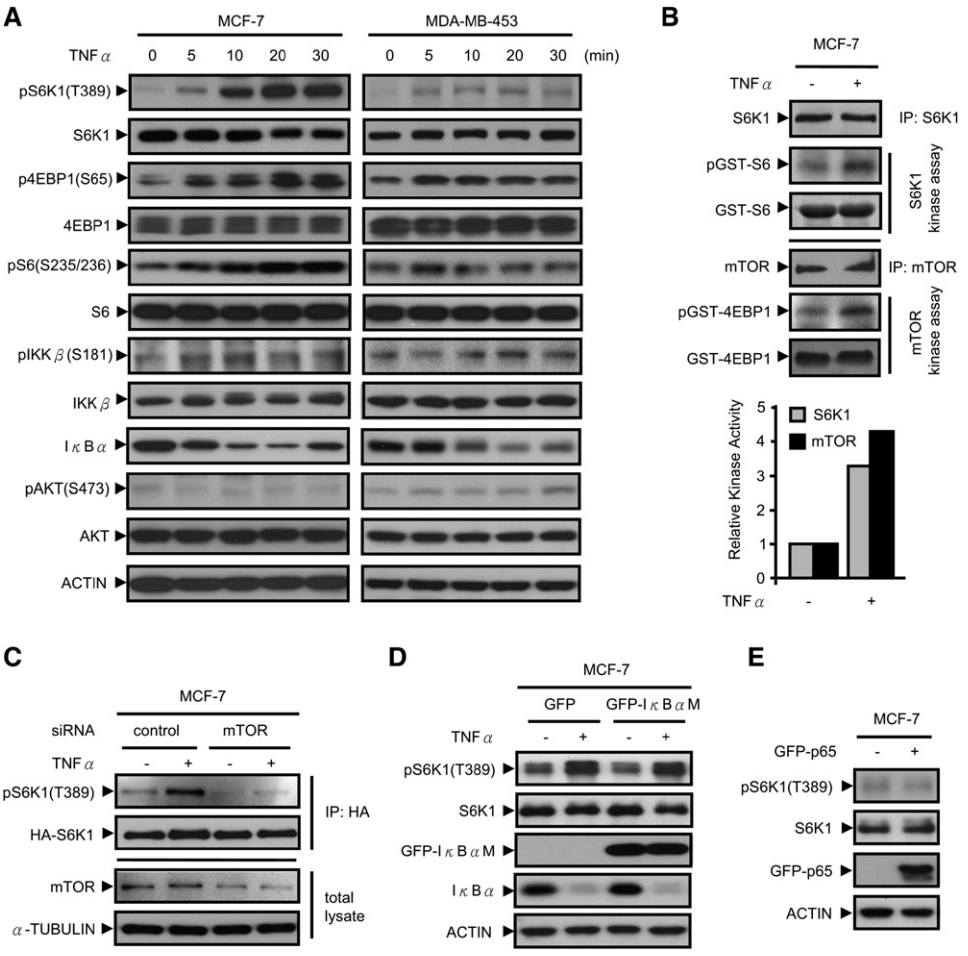


Figure 1. TNFα Activates the mTOR Pathway

(A) TNFα induced pS6K1(T389) and p4EBP1(S65) in MCF-7 and MDA-MB-453. Cells were serum-starved overnight and then treated with 20 ng/ml TNFα in a time-course study.

(B) TNFα increased the kinase activities of S6K1 and mTOR. MCF-7 cells were serum-starved overnight and then treated with 20 ng/ml TNFα for 30 min. Endogenous S6K1 and mTOR were immunoprecipitated and incubated with GST-S6 and GST-4EBP1 fusion proteins for 30 min, respectively.

(C) Knockdown of mTOR by mTOR siRNA downregulated pS6K1(T389) in MCF-7 cells. MCF-7 cells were cotransfected with HA-tagged S6K1 and mTOR siRNA, serum-starved overnight, and then treated with 20 ng/ml TNFα for 30 min.

(D) Inhibition of NF-κB by IκBαM did not impair TNFα-induced mTOR activation.

(E) NF-κB p65 did not increase pS6K1(T389).

were similar to TNFα-induced mTOR activation in their responsive cells MCF-7 and mouse RAW264.7 macrophages, respectively (Figure 3A). Furthermore, siRNA-mediated knockdown of IKKβ abrogated IL-1β- and LPS-induced pS6K1(T389) (Figures 3B and 3C). These observations supported the notion that mTOR activity may be regulated by several different inflammatory cytokines via IKKβ.

The mTOR pathway integrates signals from mitogenic growth factors, nutrients, stress, and cellular energy level to promote protein synthesis and cell growth (Fingar and Blenis, 2004; Hay and Sonenberg, 2004). Many signaling molecules, including IGF1, EGF, WNT3a (Inoki et al., 2006), glucose, and amino acids, can activate the mTOR pathway. To determine how the degree of mTOR activation in response to TNFα compared with the degree of mTOR activation in response to other agonists, we examined pS6K1(T389) induction by TNFα, IGF1, EGF, WNT3a, glucose, and amino acids, and found that TNFα-mediated mTOR activation was similar to mTOR activation by IGF1, EGF, WNT3a, and glucose under physiological conditions (Figure 3D). To further address whether IKKβ is required for other agonists-mediated mTOR activation, we examined these agonists-mediated mTOR activation in both *Ikkβ*-deficient MEFs and IKKβ-knockdown MCF-7 cells. Neither *Ikkβ* knockout nor IKKβ knockdown had significant effects on IGF1-, EGF-, or WNT3a-induced pS6K1(T389) (Figures 3E and 3F), indicating that IKKβ is specifically required for inflammatory cytokines—but not mitogens—induced mTOR activation.

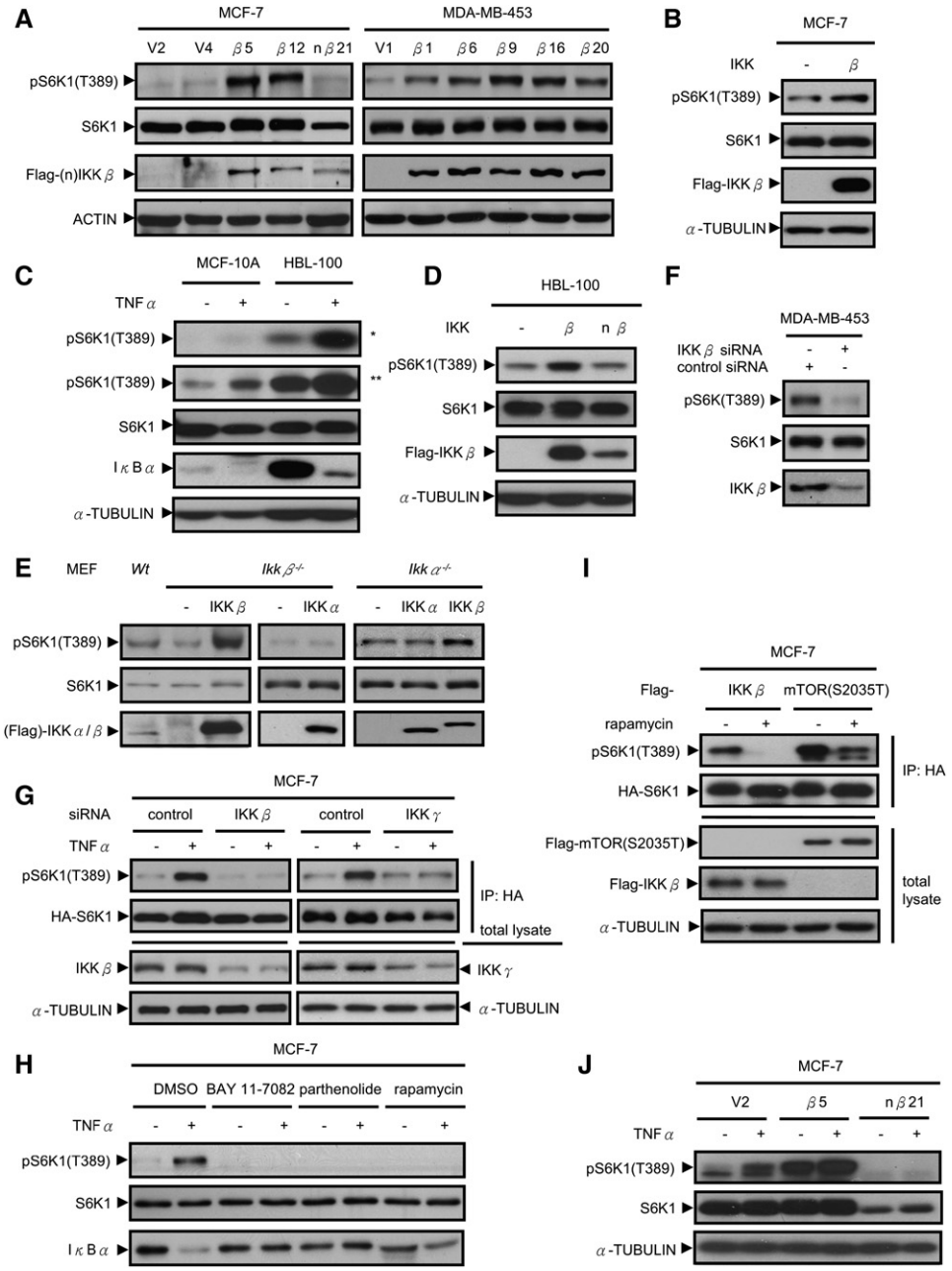


Figure 2. IKKβ is Required for TNFα-Mediated mTOR Activation

(A) Higher levels of pS6K1(T389) were observed in IKKβ (β) than in vector control (V) or nIKKβ (nβ) stable transfectants.

(B) Transient transfection of IKKβ increased pS6K1(T389) in MCF-7 cells.

(C) TNFα induced pS6K1(T389) and p4EBP1(S65) in MCF-10A and HBL-100 cells. Asterisks are as follows: *, short exposure; **, long exposure.

(D) Transient transfection of IKKβ upregulated pS6K1(T389) in HBL-100 cells.

(E) pS6K1(T389) was lower in *Ikkβ*$^{-/-}$ MEFs than in wild-type (*Wt*) MEFs and was restored by re-introduction of IKKβ but not IKKα.

(F) Knockdown of IKKβ by IKKβ siRNA downregulated pS6K1(T389) in MDA-MB-453 cells.

(G) siRNA-mediated knockdown of IKKβ and IKKγ inhibited TNFα-induced pS6K1(T389) in MCF-7 cells. MCF-7 cells were cotransfected with HA-tagged S6K1 and either IKKβ or IKKγ siRNA and then treated with 20 ng/ml TNFα for 30 min.

(H) The IKKβ inhibitors BAY 11-7082 (40 μM, 45 min pretreatment) and parthenolide (80 μM, 45 min pretreatment) and the mTOR inhibitor rapamycin (50 nM, 3 h pretreatment) blocked TNFα-induced pS6K1(T389) in MCF-7 cells.

(I) Rapamycin abolished pS6K1(T389) in IKKβ-transfected MCF-7 cells but not in mTOR(S2035T)-transfected MCF-7 cells. MCF-7 cells were cotransfected with HA-tagged S6K1 and either IKKβ or rapamycin-insensitive mTOR(S2035T) and then treated with 50nM rapamycin for 1 h.

(J) TNFα-induced mTOR activation was impaired in MCF-7 nIKKβ stable transfectant.

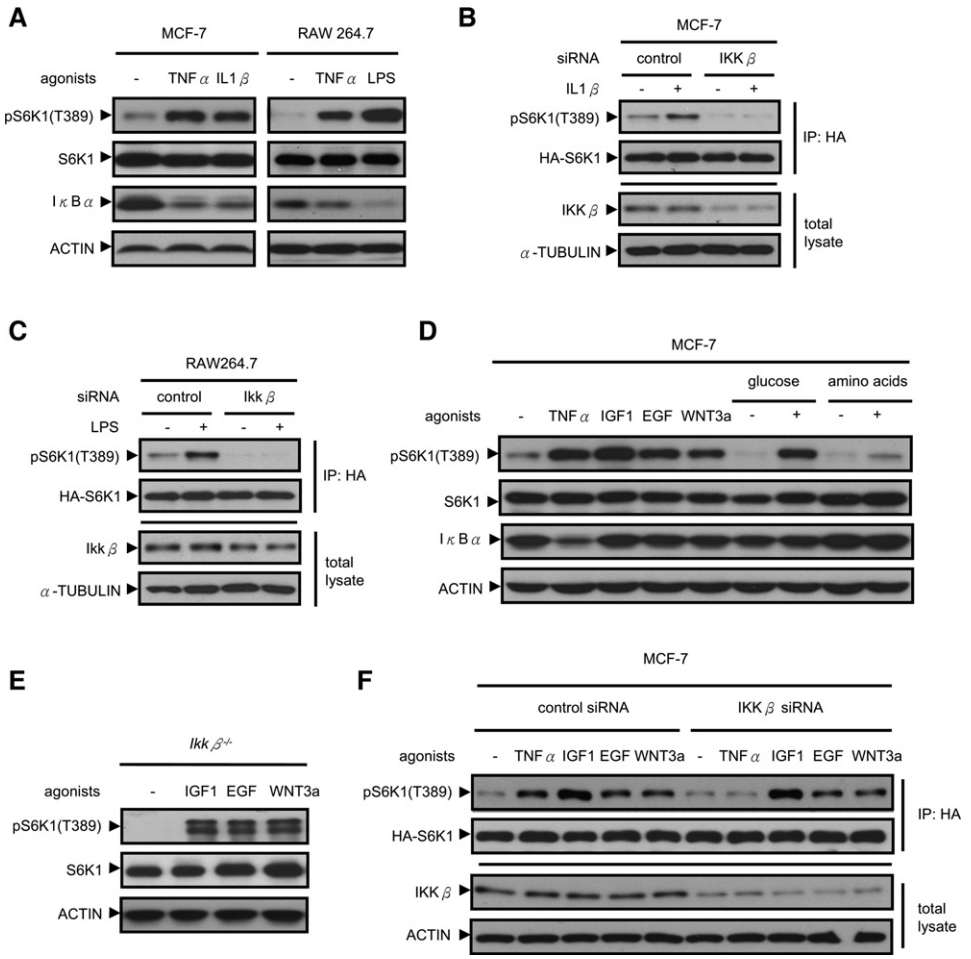


Figure 3. Induction of mTOR Signaling by Inflammatory Cytokines

(A) TNFα, IL1β, and LPS induced pS6K1(T389) in MCF-7 cells and RAW264.7 mouse macrophages. Cells were serum-starved overnight and then treated with 20 ng/ml TNFα, 20 ng/ml IL1β, and 10 ng/ml LPS for 30 min, respectively.

(B) siRNA-mediated knockdown of IKKβ inhibited IL1β-induced pS6K1(T389) in MCF-7 cells. MCF-7 cells were cotransfected with HA-tagged S6K1 and IKKβ siRNA and then treated with 20 ng/ml IL1β for 30 min.

(C) siRNA-mediated knockdown of Ikkβ inhibited LPS-induced pS6K1(T389) in RAW264.7 mouse macrophage. RAW264.7 cells were cotransfected with HA-tagged S6K1 and Ikkβ siRNA and then treated with 10 ng/ml LPS for 30 min.

(D) pS6K1(T389) induction by TNFα (20 ng/ml) was similar to pS6K1(T389) induction by IGF1 (20 ng/ml), EGF (20 ng/ml), WNT3a (20 ng/ml), glucose (15 mM), and amino acids.

(E) No significant effects on IGF1-, EGF-, or WNT3a-induced pS6K1(T389) in *Ikkβ*$^{-/-}$ MEFs.

(F) pS6K1(T389) induction by IGF1 (20 ng/ml), EGF (20 ng/ml), and WNT3a (20 ng/ml) was only slightly inhibited in IKKβ-knockdown MCF-7 cells.

IKKβ Physically Interacts with TSC1

Our finding that IKKβ activates the mTOR pathway prompted us to investigate the mechanism by which IKKβ activates mTOR. Since the TSC1/TSC2 complex and RHEB are the upstream molecules responsible for the regulation of mTOR activity, we investigated whether the regulation of mTOR function by TNFα/IKKβ signaling is through the TSC complex. TNFα induced mTOR activation in *Tsc1*$^{+/+}$ and *Tsc2*$^{+/+}$ MEFs but not in *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$ MEFs in which the mTOR is constitutively activated (Figure 4A), and this effect was recovered by re-expression of TSC1 and TSC2 in *Tsc1*$^{-/-}$ and *Tsc2*$^{-/-}$ MEFs, respectively (Figure S3), indicating that TNFα-dependent mTOR activation requires TSC1/2 complex. Increased pS6K1(T389) levels as a result of transient expression of IKKβ in *Tsc1*$^{+/+}$ but not in *Tsc1*$^{-/-}$ MEFs (Figure 4B) further indicated that TSC1 is involved in TNFα/IKKβ-mediated mTOR activation. Analysis of amino acid sequences revealed that TSC1, but not TSC2 or RHEB, has a putative IKKβ phosphorylation site at Ser511 (Figure 4C). In addition, cotransfection of TSC1 and IKKβ showed that IKKβ caused the accumulation of a slow-migrating form of TSC1 that was entirely eliminated by treatment with calf intestinal alkaline phosphatase (Figure S4), indicating that the mobility shift was caused by phosphorylation of TSC1. Coimmunoprecipitation experiments further

showed that IKKβ but not IKKα or IKKγ physically associated with TSC1 (Figure S5). This interaction between IKKβ and TSC1 was also observed with endogenous IKKβ and TSC1 using specific antibodies to IKKβ and TSC1 (Figure 4D), suggesting that IKKβ physically associates with TSC1. In vitro pull-down assay further suggested a direct association between IKKβ and TSC1 (Figure 4E). Since IKKγ was required for TNFα-induced mTOR activation (Figure 2G, right panel), we investigated whether IKKγ plays a role in the association between IKKβ and TSC1. IKKγ-knockdown MCF-7 cells were used for a coimmunoprecipitation assay and the results indicated that IKKγ was not necessary for the association between IKKβ and TSC1 (Figure S6). This result was consistent with the fact that IKKγ is only required for TNFα-induced mTOR activation by IKKβ (Figure 2G, right panel) whereas the association between IKKβ and TSC1 is direct (Figure 4E) and independent of IKKγ (Figure S6).

IKKβ Phosphorylates TSC1 Predominantly at Ser487 and Ser511 In Vivo and In Vitro

Given the physical interaction between IKKβ and TSC1 and the mobility shift induced by IKKβ in TSC1, we examined whether TSC1 is a physiological substrate of IKKβ. In vitro kinase assays demonstrated that the TSC1 domain 301-600 was strongly phosphorylated by IKKβ, whereas other domains of TSC1 were not phosphorylated by IKKβ under the same conditions (Figure S7A, lanes 1-4). Further analysis revealed that the phosphorylation sites were located between residues 401-500 and 501-600 of TSC1 (Figure S7B). The phosphorylation of TSC1 was similar in intensity to that of IκBα, a well-known substrate of IKKβ. Mass spectrometry analysis of GST-TSC1 fragments showed that Ser487 and Ser511 were phosphorylated by IKKβ (Figures S8A and S8B). Mutation of either of these Ser residues to Ala (S487A or S511A) abolished the phosphorylation of the TSC1 fragments by IKKβ immunocomplexes (Figure 4F) and purified IKKβ kinase (data not shown), suggesting that IKKβ directly phosphorylates TSC1 at both Ser487 (a nontraditional phosphorylation site) and Ser511 (an orthodox phosphorylation site) in vitro.

To assess whether these phosphorylations occur in vivo, mass spectrometry analysis was performed. This analysis showed that TSC1 Ser487 and Ser511 were phosphorylated by IKKβ in vivo (Figures S8C and S8D). To investigate TSC1 phosphorylation by IKKβ in vivo, we raised rabbit polyclonal antibodies to TSC1 phosphorylated at Ser487 [pTSC1(S487)] or Ser511 [pTSC1(S511)] in collaboration with Bethyl Laboratories and used these antibodies to examine the phosphorylation status of TSC1 in vivo. These antibodies specifically recognized the phosphorylation of Ser487 and Ser511 by IKKβ but not by nIKKβ and failed to detect any phosphorylation when TSC1(SSAA) (in which both IKKβ phosphorylation sites were replaced with Ala to abolish the phosphorylation by IKKβ) was cotransfected with IKKβ (Figure 4G). Consistent with our earlier finding that IKKβ but not IKKα interacted with TSC1 (Figure 4D), Ser487 and Ser511 were phosphorylated by IKKβ but not IKKα (Figure 4H). Studies of the effect of TNFα on TSC1 phosphorylation over time showed that pTSC1(S511) was induced by TNFα between 10 and 30 min and then declined (Figure 4I). Interestingly, pTSC1(S487) induced by TNFα lasted longer than 1 h. The difference in the kinetics of TNFα induction of pTSC1(S487) and pTSC1(S511) implied that these two phosphorylations might be regulated by distinct dephosphorylation mechanisms. Notably, TNFα-induced pTSC1(S487) and pTSC1(S511) were abrogated by the IKKβ inhibitor BAY 11-7082 (Figure 4J) and by IKKβ siRNA (Figure S9A), suggesting that TNFα-induced pTSC1(S487) and pTSC1(S511) are dependent on IKKβ. Furthermore, the anti-pTSC1(S511) antibody but not the anti-TSC1(S487) antibody could be used for immunostaining (Figure S9B and data not shown). After TNFα stimulation, endogenous pTSC1(S511) was recognized by the antibody; this phosphorylation signal was abrogated by the IKKβ inhibitor BAY 11-7082 and by the blocking phosphopeptide but not by the control nonphosphopeptide (Figure S9B). Taken together, our results showed that TSC1 is phosphorylated by IKKβ at Ser487 and Ser511 in vitro and in vivo and that IKKβ is required for TNFα-induced phosphorylation of TSC1.

Phosphorylation of TSC1 by IKKβ Suppresses TSC1 Function

On the basis of our observations that IKKβ phosphorylates TSC1 and induces pS6K1(T389), we asked whether IKKβ phosphorylation leads to functional inactivation of TSC1, thereby activating mTOR. To address this issue, we generated six TSC1 mutants: TSC1(S487A), TSC1(S511A), TSC1(SSAA), TSC1(S487D), TSC1(S511D), and TSC1(SSDD). In TSC1(SSDD), both IKKβ phosphorylation sites were replaced with Asp to mimic the phosphorylation of TSC1 by IKKβ. We tested the effects of these mutants on mTOR-dependent S6K1 phosphorylation at T389 in *Tsc1*-deficient MEFs. The pS6K1(T389) levels in cells transfected with TSC1 Asp mutants were higher than those in cells transfected with TSC1 Ala mutants (Figure 5A). Moreover, TNFα could induce pS6K1(T389) in TSC1(WT) but not in TSC1(SSAA)-expressed $Tsc1^{-/-}$ MEFs (Figure 5B). We further investigated whether either one of the these two serines (Ser487 and Ser511) may also be involved in TNFα-induced mTOR activation. These results showed that mutation of either one of these two sites only partially inhibited TNFα-induced pS6K1(T389), suggesting that both Ser487 and Ser511 were involved in regulation of TNFα-induced mTOR activation (Figure 5C).

Next, we investigated the underlying mechanisms leading from TSC1 phosphorylation to mTOR activation. We noticed that TSC1(SSDD) was consistently expressed at a lower level than TSC1(WT) and TSC1(SSAA) when the same amount of DNA was transfected. To ensure the same level of TSC1 protein expression, the DNA amount of TSC1(SSDD) used for transient transfection was greater than the DNA amount of TSC1(WT) and TSC1(SSAA)

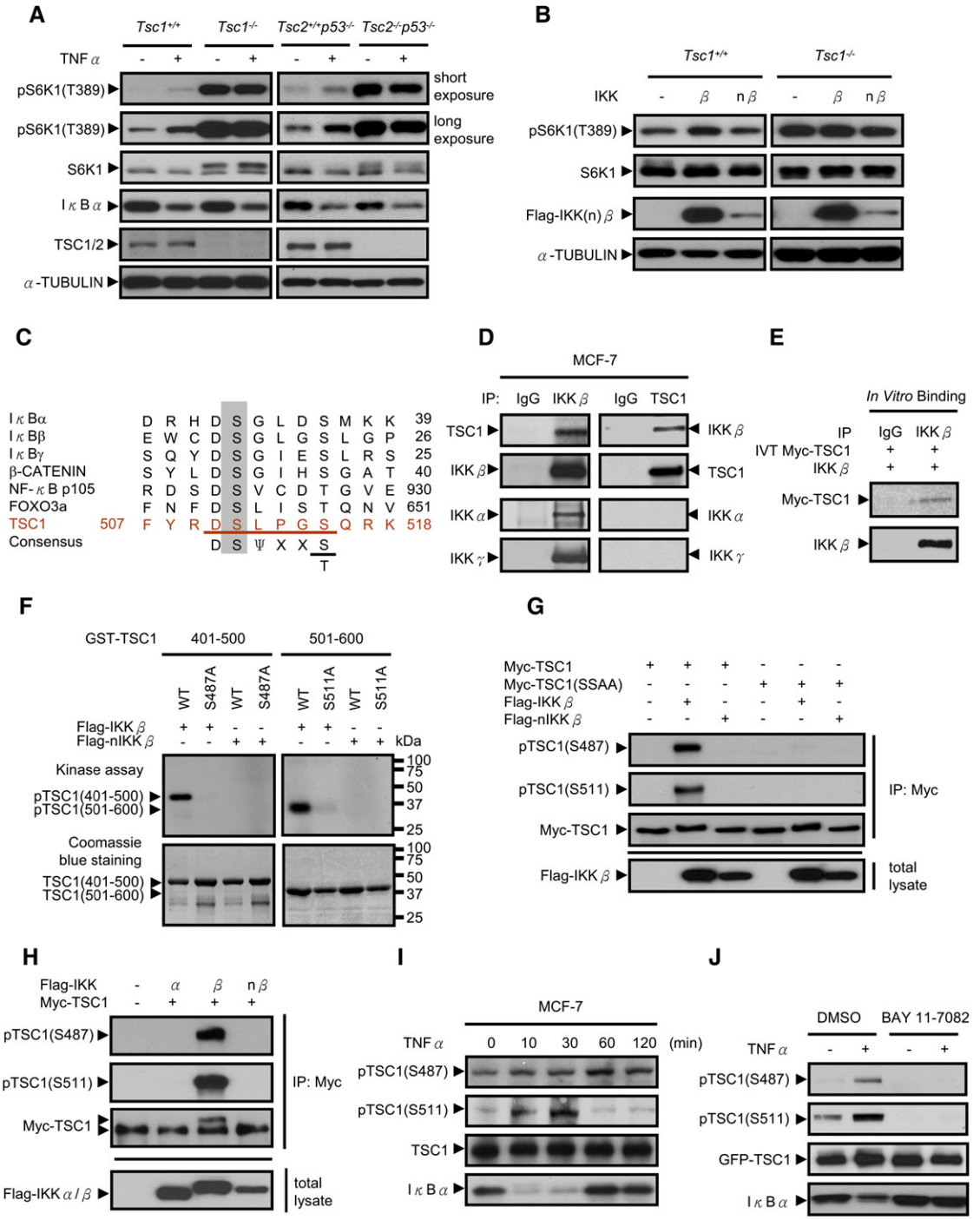


Figure 4. IKKβ Phosphorylates TSC1 In Vivo and In Vitro

(A) TNFα induced mTOR activation in wild-type but not in *Tsc1*$^{-/-}$ or *Tsc2*$^{-/-}$ MEFs. Cells were serum-starved overnight and then treated with 20 ng/ml TNFα for 30 min.

(B) IKKβ induced mTOR activation in *Tsc1*$^{+/+}$ but not in *Tsc1*$^{-/-}$ MEFs.

(C) TSC1 has a putative IKKβ phosphorylation site at Ser511 (DS^{511}LPGS). S, serine; T, threonine; ψ, hydrophobic amino acid; X, any amino acid.

(D) Endogenous TSC1 interacted with IKKβ but not IKKα or IKKγ. Lysates of MCF-7 cells were analyzed by reciprocal coimmunoprecipitation and immunoblotting.

(E) TSC1 interacted directly with IKKβ. In vitro transcribed TSC1 proteins were incubated with recombinant IKKβ proteins and then pulled down by antibody to IKKβ. IVT, in vitro transcription and translation.

(Figures 5A and 5B). We tested whether phosphorylation by IKKβ affects TSC1 protein stability and thus contributes to the inactivation of TSC1 function. Treatment with TNFα increased the ubiquitination level of TSC1 and TSC2 (Figure S10A), and the ubiquitination level of TSC1(SSDD) was higher than that of TSC1(SSAA) (Figure S10B). Treatment with cycloheximide to inhibit protein synthesis further revealed that the half-life of TSC1(SSDD) (10 h) was shorter than that of TSC1(WT) (22 hr) or TSC1(SSAA) (24 hr) (Figure 5D). Although the shorter half-life of the phosphorylated TSC1 by IKKβ reflects the fate of faster degradation, it might not have a major role in TNFα-induced mTOR activation, which occurs within 30 min (Figure 1A).

Since TSC1 stabilizes TSC2 by preventing the association between TSC2 and HERC1 ubiquitin ligase, enhances TSC2 GAP function, and further inhibits mTOR activity (Chong-Kopera et al., 2006), we investigated whether phosphorylation of TSC1 by IKKβ inhibits TSC1/TSC2 complex formation. Indeed, TNFα treatment, which induced IKKβ to phosphorylate TSC1, interfered with TSC complex formation (Figure 5E). Consistent with this finding, TSC1(SSDD) had a weaker interaction with TSC2 than did TSC1(WT) or TSC1(SSAA) (Figure S10C). Taken together, these results suggested that the IKKβ-dependent phosphorylation of TSC1 enhances dissociation of TSC1/TSC2 complex, which in turn induces mTOR activation.

Recently, the membrane-associated TSC1/TSC2 complex has been recognized to be biologically active and able to impede RHEB function (Potter et al., 2002). We therefore examined whether phosphorylation by IKKβ affects the membrane localization of TSC1 and TSC2. The membrane-bound TSC1 did not change its subcellular localization in response to TNFα (Figure 5F). Consistently, both TSC1(SSAA) and TSC1(SSDD) mutants localized at the membrane fractions (Figure S10D). Interestingly, we observed a small fraction of the membrane-associated TSC2 migrated to the cytosol after TNFα treatment (Figure 5F). This may be due to the fact that phosphorylation of TSC1 by IKKβ inhibits the association of TSC1 with TSC2 and thereby promotes a change in the subcellular localization of TSC2. Collectively, our results indicated that phosphorylation of TSC1 by IKKβ interferes with the association of TSC1 with TSC2, which alters TSC2 membrane localization and activates the mTOR pathway.

Repression of TSC1 Promotes Tumor Angiogenesis

We next asked whether phosphorylation by IKKβ also affects the tumor suppressor function of TSC1. In vivo tumorigenesis assays revealed that the expression of both TSC1(WT) and TSC1(SSAA) profoundly antagonized tumorigenesis of 4T1 cells (82% reduction in tumor volume compared with the vector control), whereas TSC1(SSDD) had little effect (Figure 6A). In comparison with TSC1(WT), the stronger tumor suppression effect of TSC1(SSAA) could be detected in the first three weeks (Figure 6A and Table S1). To investigate whether activation of the mTOR pathway plays a role in IKKβ-induced tumorigenesis, we treated mice bearing 4T1-IKKβ, 4T1-nIKKβ, 4T1-TSC1(SSDD), and 4T1-TSC1(SSAA) tumors with the mTOR inhibitor rapamycin. Rapamycin inhibited 4T1-IKKβ-mediated (Figure 6B) and 4T1-TSC1(SSDD)-mediated tumor progression (Figure 6C). Furthermore, TSC1(SSAA), which suppressed mTOR activity (Figures 5A and 5B), strongly inhibited IKKβ-mediated tumor progression (Figure 6D), while TSC1(WT), which could still respond to TNFα/IKKβ-induced phosphorylation and inactivation, only partially interfered with IKKβ-induced tumorigenesis, indicating that activation of the TSC/mTOR pathway is required for IKKβ-mediated tumor development.

Since loss of Tsc1 leads to upregulation of Vegf expression, which is dependent on the mTOR pathway, and this event contributes to the vascular nature of TSC lesions (El-Hashemite et al., 2003), we assessed the mean density of blood vessels from 4T1 tumors to elucidate whether TSC1(SSDD) loses antiangiogenic activity and therefore lacks tumor suppressor function. Indeed, the mean blood vessel density in 4T1-TSC1(SSDD) tumors was significantly higher than the mean blood vessel density in 4T1-TSC1(WT) and 4T1-TSC1(SSAA) tumors (Figure 6E), suggesting that phosphorylation by IKKβ impairs the antiangiogenic function of TSC1. A similar result was found in mice bearing 4T1 tumors injected intratumorally with various TSC1 constructs complexed with DOTAP:Chol liposome (Figure S11A). The notion that IKKβ impairs the antiangiogenic function of TSC1 was further supported by two in vitro angiogenesis assays, the human umbilical vein endothelial cell (HUVEC) tube formation and migration assays: TSC1(SSAA) stable transfectants had a stronger ability than did TSC1(SSDD) transfectants to inhibit HUVEC tube formation and migration (Figures 6F and Figure S11B).

To further address how phosphorylated TSC1 loses its antiangiogenic ability to suppress VEGF, we measured VEGF secretion from stable transfectants. TSC1(SSAA) transfectants, but not TSC1(SSDD) transfectants, inhibited VEGF secretion (Figure 6G). Similar results were found in MDA-MB-453 cells transiently transfected with

(F) The IKKβ phosphorylation sites Ser487 and Ser511 were identified by in vitro kinase assays in which IKKβ and nIKKβ immunocomplexes from HEK293 cells were incubated with the indicated GST-TSC1 fusion proteins. WT, wild-type.

(G) Characterization of antibodies to pTSC1(S487) and pTSC1(S511). Myc-tagged TSC1(wild-type) and TSC1(SSAA) were cotransfected with Flag-tagged IKKβ or nIKKβ, immunoprecipitated, and analyzed with antibodies to pTSC1(S487) and pTSC1(S511).

(H) IKKα did not phosphorylate TSC1 at Ser487 or Ser511. Myc-tagged TSC1(wild-type) was cotransfected with Flag-tagged IKKα, IKKβ, or nIKKβ, immunoprecipitated, and analyzed with antibodies to pTSC1(S487) and pTSC1(S511).

(I) TNFα induced phosphorylation of endogenous TSC1 at Ser487 and Ser511 in MCF-7 cells.

(J) The IKKβ inhibitor BAY 11-7082 inhibited TNFα-induced phosphorylation of TSC1 at Ser487 and Ser511.

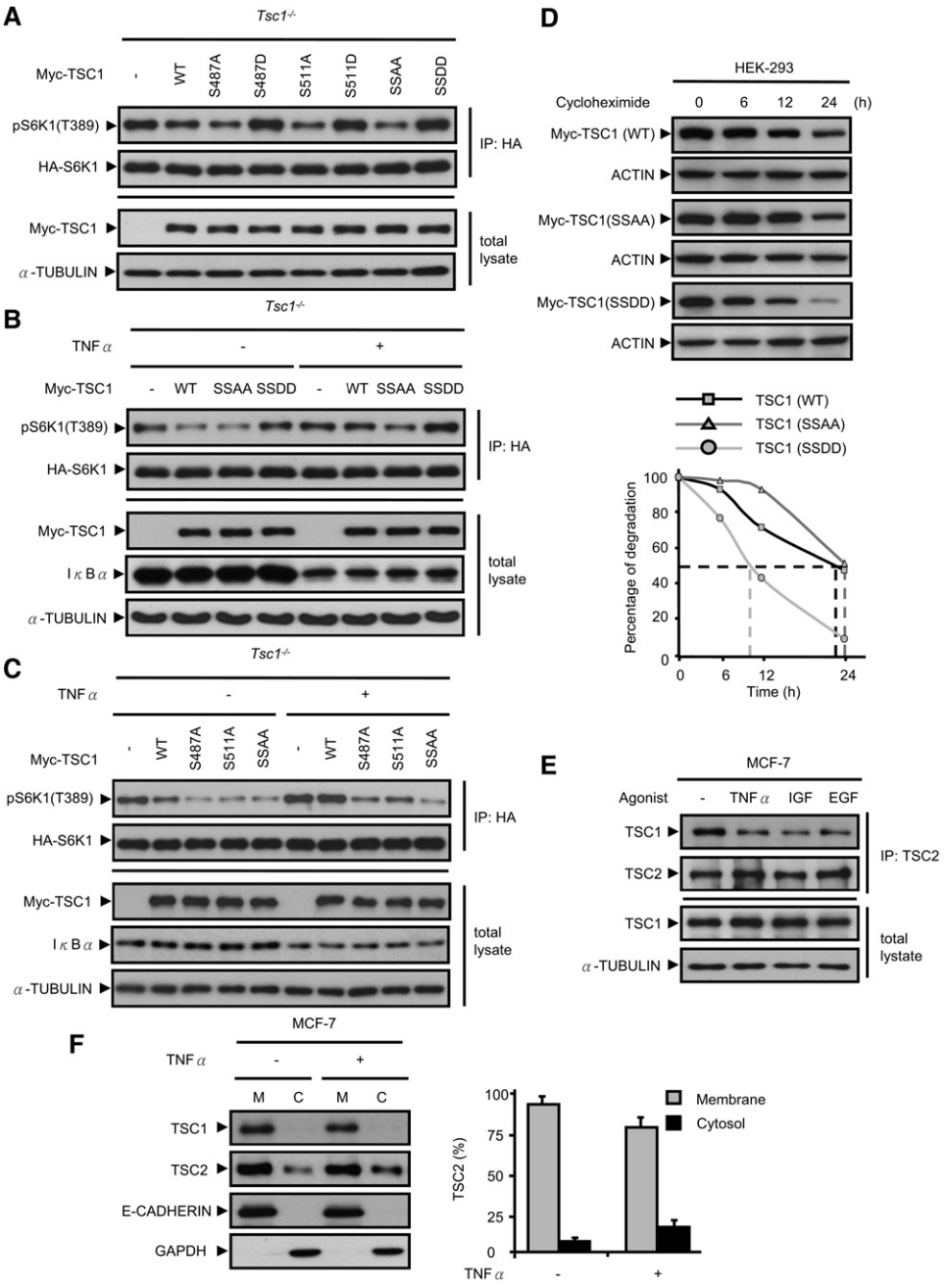


Figure 5. IKKβ Phosphorylation Suppresses TSC1 Function

(A) pS6K(T389) levels were lower in cells transfected with TSC1 Ala mutants than in cells transfected with TSC1 Asp mutants. HA-tagged S6K1 was transiently transfected into *Tsc1*-deficient MEFs with wild-type TSC1 or the indicated mutants of TSC1, and expression was analyzed by antibody to pS6K(T389) after 16 h of serum starvation. To ensure the same level of TSC1 protein expression, the DNA amount of TSC1 Asp mutants used for transfection was greater than the DNA amount of TSC1 wild-type and Ala mutants.

(B) Induction of pS6K1(T389) by TNFα was repressed in TSC1(SSAA)-transfected $Tsc1^{-/-}$ MEFs.

(C) TSC1(SSAA) strongly suppressed TNFα-induced pS6K1(T389), while TSC1(S487A) and TSC1(S511A) only had partial effects.

(D) The TSC1(SSDD) mutant was less stable than TSC1(WT) or TSC1(SSAA). The stability of wild-type and mutant TSC1 was determined by cycloheximide (100 μg/ml) treatment.

(E) TNFα disrupted the interaction between TSC1 and TSC2. MCF-7 cells were serum-starved overnight and then treated with either 20 ng/ml TNFα, 20 ng/ml IGF1, or 20 ng/ml EGF for 30 min, immunoprecipitated by antibody to TSC2, and analyzed with antibody to TSC1.

various TSC1 mutants (Figure S11C). Depletion of VEGF by VEGF-specific antibody repressed the HUVEC tube formation ability of TSC1(SSDD) to a level similar to that for the HUVEC tube formation ability of TSC1(SSAA) (Figure 6H). Moreover, the cell growth and VEGF secretion induced by TNFα were lower in TSC1(SSAA) stable transfectant than in TSC1(WT) stable transfectants (Figures S11D and S11E), and the TSC1(WT)- and TSC1(SSAA)-mediated suppression of VEGF expression and HUVEC tube formation were diminished after transfection of constitutively active S6K1(E389D3E) (Figures 6F and 6G versus Figures S11F and S11G). In addition, rapamycin strongly suppressed VEGF expression induced by IKKβ but had much less effect on VEGF expression induced by a rapamycin-insensitive mTOR (S2035T) mutant (Figure S11H). Taken together, our results suggest that TSC1 exerts an antiangiogenic effect through suppression of VEGF expression and also suggest that phosphorylation of TSC1 by IKKβ activates mTOR and then de-represses VEGF expression, impairing the antiangiogenic function of TSC1.

Clinical Relevance of pTSC1(S511) in Human Tumor Tissues

To further examine whether the aforementioned conclusions could be supported in human primary tumors, we studied the expression of pIKKβ(S181) (activated IKKβ) and pTSC1(S511) in 115 human primary breast tumor specimens by immunohistochemical staining. pIKKβ(S181) was strongly correlated with the expression of TNFα and pIκBα(S32/S36) (Figures S12A and S12B), suggesting that pIKKβ(S181) can be used to stand for both TNFα-stimulated signaling and IKKβ kinase activity. Moreover, pTSC1(S511) was detected in 29 (69%) of the 42 specimens with high pIKKβ(S181) expression but in only 22 (30%) of the 73 specimens with low pIKKβ(S181) expression, indicating that pIKKβ(S181) expression was associated with high levels of pTSC1(S511) ($p < 0.001$; Figures 7A and 7B). Consistent with this finding, pTSC1(S511) expression was also strongly associated with pS6K1(T389) and VEGF expression ($p < 0.001$; Figures 7A and 7B). We also performed kinase assays and immunoblotting using 10 freshly prepared human breast tumor lysates to validate the relationships between IKKβ kinase activity, pTSC1(S511) expression, and pS6K1(T389) expression. Consistent with the findings on immunohistochemical staining, IKKβ kinase activity was significantly associated with pTSC1(S511) and pS6K1(T389) by immunoblotting [$p < 0.01$ for IKKβ kinase activity and pTSC1(S511); $p < 0.05$ for IKKβ kinase activity and pS6K1(T389); $p < 0.01$ for pTSC1(S511) and pS6K1(T389); Figures 7C and 7D]. To investigate whether the association between pTSC1(S511) and pIKKβ(S181) also occurs in other tumor types, human tumor tissue arrays were examined. The results demonstrated that pTSC1(S511) expression was associated with pIKKβ(S181) not only in breast cancer but also in many other kinds of cancers, including colon and liver cancers (Figure S12C and Table S2). In addition, the percentage of pTSC1(S511)-positive normal tissues (24.8%) was significantly lower than the percentage of pTSC1(S511)-positive tumor tissues (69.7%) ($p < 0.001$; Figure S12C and Tables S2 and S3), suggesting that phosphorylation of TSC1 by IKKβ is pathologically relevant in multiple human tumors. We next analyzed the expression of pTSC1(S511) and pS6K1(T389) in breast tumor tissues and correlated the findings with patient survival data. The Kaplan-Meier overall survival curves showed that high pTSC1(S511) and pS6K1(T389) levels were associated with poor survival. Moreover, the combination of pTSC1(S511) and pS6K1(T389) was a better predictor of survival than was either factor alone ($p < 0.01$ versus $p < 0.05$) (Figure 7E). Taken together, the tumor immunohistochemical staining data further strengthened the notion that phosphorylation of TSC1 by IKKβ promotes S6K1 activation and VEGF production and is associated with poor clinical outcome of breast cancer patients.

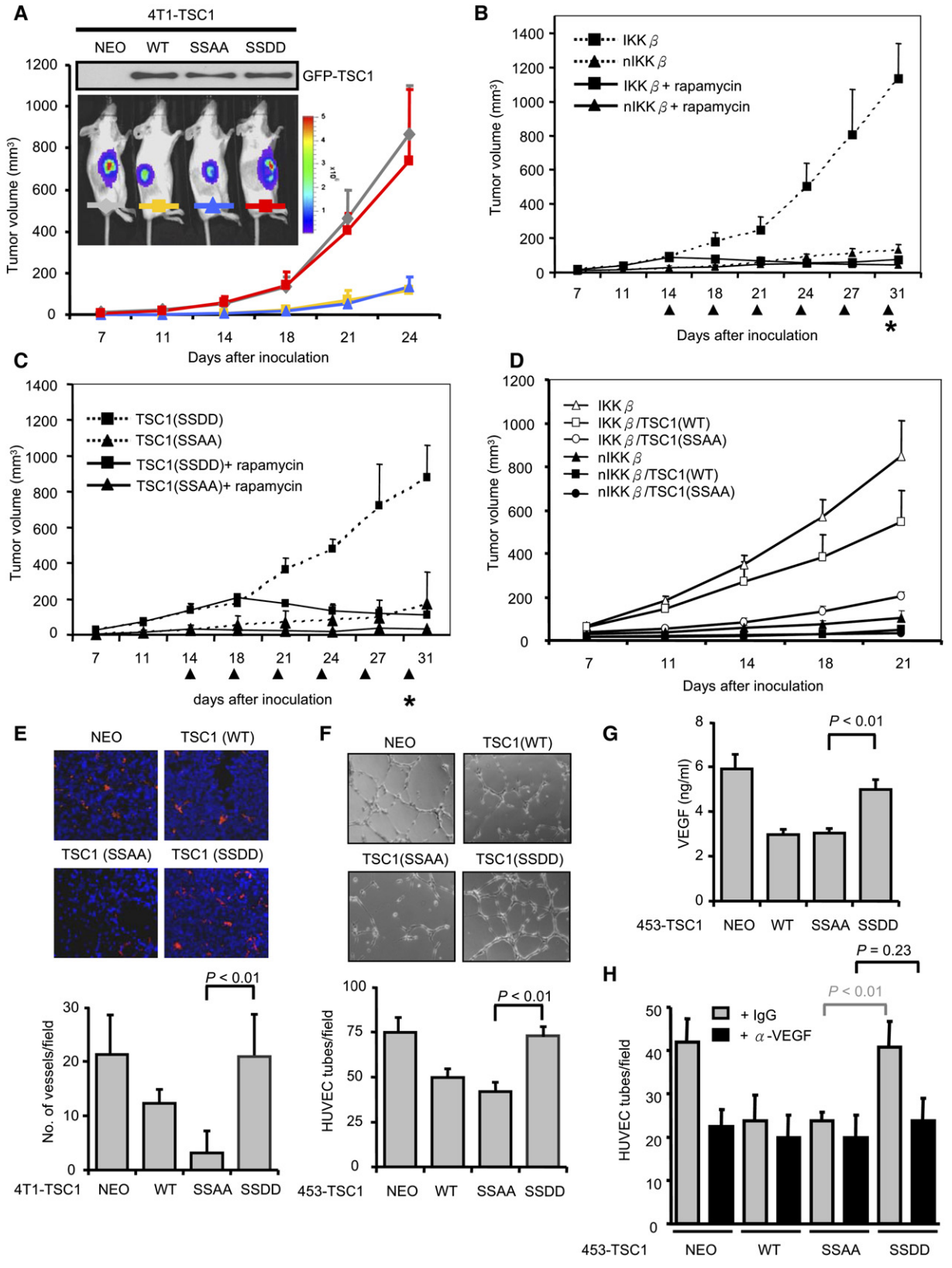
DISCUSSION

The link between inflammation and tumor progression has been suspected for about two hundred years, and accumulating evidence supports a tumor-promoting role of inflammation, brilliantly described by Balkwill and Mantovani as follows: "If genetic damage is the 'match that lights the fire' of cancer, some types of inflammation may provide the 'fuel that feeds the flames'" (Balkwill and Mantovani, 2001). The proinflammatory cytokines and chemokines produced in the tumor microenvironment, such as TNFα, IL-1, IL-6, and IL-8, enhance cell proliferation, cell survival, cell migration, and tumor angiogenesis, thereby promoting tumor development (Karin and Greten, 2005). Although accumulating evidence suggests that angiogenesis plays a role in inflammation-mediated tumorigenesis, the detailed mechanisms by which inflammation facilitates tumor development remain to be further investigated.

Activation of the mTOR Pathway by TNFα

Dysregulation of TNFα signaling pathway contributes to the development of human cancers due to enhanced IKK activity and constitutive NF-κB activation. Here, we showed that TNFα also stimulates mTOR signaling, which is known to upregulate protein translation including VEGF, through phosphorylation and inactivation of TSC1 by IKKβ in breast cancers. This regulation may be relevant in both the epithelial tumors and hematological malignancies, and

(F) TSC1 remained membrane bound in response to TNFα, whereas some portion of TSC2 migrated to the cytosol. The membrane and cytosolic fractions of serum-starved MCF7 cells that were stimulated with 20ng/ml TNFα for 30 min were analyzed by immunoblotting with antibodies to TSC1 and TSC2. E-CADHERIN was used as the membrane marker, and GAPDH was used as the cytosolic marker. M, membrane fraction; C, cytosolic fraction. The plots represent the mean of three independent experiments and error bars represent SD.


A
4T1-TSC1
NEO WT SSAA SSDD
GFP-TSC1
Tumor volume (mm³)
Days after inoculation
B
IKKβ
nIKKβ
IKKβ + rapamycin
nIKKβ + rapamycin
Tumor volume (mm³)
Days after inoculation
C
TSC1(SSDD)
TSC1(SSAA)
TSC1(SSDD)+ rapamycin
TSC1(SSAA)+ rapamycin
Tumor volume (mm³)
days after inoculation
D
IKKβ
IKKβ/TSC1(WT)
IKKβ/TSC1(SSAA)
nIKKβ
nIKKβ/TSC1(WT)
nIKKβ/TSC1(SSAA)
Tumor volume (mm³)
Days after inoculation
E
NEO
TSC1 (WT)
TSC1 (SSAA)
TSC1 (SSDD)
P < 0.01
No. of vessels/field
4T1-TSC1 NEO WT SSAA SSDD
F
NEO
TSC1(WT)
TSC1(SSAA)
TSC1(SSDD)
P < 0.01
HUVEC tubes/field
453-TSC1 NEO WT SSAA SSDD
G
P < 0.01
VEGF (ng/ml)
453-TSC1 NEO WT SSAA SSDD
H
P = 0.23
P < 0.01
+ IgG
+ α-VEGF
HUVEC tubes/field
453-TSC1 NEO WT SSAA SSDD

contribute to not only tumor progression but also tumor metastasis as VEGF-regulated angiogenesis is a key factor for metastasis. On the basis of our findings, we propose a model in which elevated TNFα from macrophages and tumor cells upregulates the mTOR pathway through inactivation of TSC1 by IKKβ, leading to increasing VEGF expression and promotion of tumor angiogenesis (Figure 7F). It is worthwhile to mention that TNFα stimulates AKT activation dependent upon cell types (Delhase et al., 2000; Ghosh et al., 2006; Ozes et al., 2001). Thus, it may be possible that TNFα could activate both IKKβ and AKT to inactivate TSC1 and TSC2, respectively, and then results in mTOR activation in certain cell types. Interestingly, a recently published study by T. Hunter and colleagues demonstrated the attenuation of NF-κB activation due to constitutive mTOR activation in *TSC*-derived tumors, which might explain why tuberous sclerosis are weakly malignant (Ghosh et al., 2006). The mTOR activity is inducible by cytokines and the level is modest compared with the constitutively activated mTOR activity in the *Tsc* null cells (Figure 4A). Since TNFα-induced NF-κB activation is regulated normally in the cells with functional TSC1/TSC2, we speculate that the impairment of NF-κB activation might only occur in the tumors with deficient or mutated TSC1/TSC2 in which the mTOR is constitutively and highly activated, but not in tumors with normal TSC function. Integrating these findings may provide a new view on the therapeutic intervention for inflammation-mediated cancers by using IKK inhibitors or combining NF-κB inhibitors and rapamycin.

TNFα-activated IKK Complex and Individual Functionality of IKKβ

The requirement of IKK complex for activation of NF-κB has been studied for many years. Although dogma holds that IKK kinase is an IκB kinase that is responsible for the phosphorylation of IκBs, growing evidence suggests that IKKα and IKKβ also play other roles in regulating physiological reactions. For instance, IKKα shuttles from cytoplasm to nucleus and phosphorylates HISTONE H3 and CBP (Anest et al., 2003; Huang et al., 2007; Yamamoto et al., 2003), and IKKβ can phosphorylate and modulate the functions of several cytosol proteins, including β-CATENIN (Lamberti et al., 2001), SRC-3 (Wu et al., 2002), FOXO3a (Hu et al., 2004), CYLD (Lee et al., 2004), and DOK1 (Reiley et al., 2005). Curiously, our study of the interaction between TSC1 and the IKK complex revealed that only IKKβ, not IKKα, associates with TSC1, suggesting that TSC1-associated IKKβ might be a free form of IKKβ. However, TSC1 phosphorylation and S6K1 phosphorylation were increased after TNFα stimulation, and knockdown of IKKγ, a component in the IKK complex known to be required for activation of IKKβ, abolished TNFα-induced mTOR activation. These findings suggested that the complete IKK complex is required for TNFα-induced TSC1 phosphorylation and mTOR activation. Together with the existing knowledge and our results that IKKβ (but not IKKα and IKKγ) associates with TSC1, and the basal level of pS6K1(T389) is reduced in both IKKβ-knockdown and IKKβ inhibitor-treated cells (Figures 2G and 2H) but not IKKγ-knockdown cells (Figure 2G), an interesting model emerges: in response to extracellular cytokines such as TNFα, the IKK complex is activated and then phosphorylates IκB inhibitors. However, some portions of activated IKK kinases might dissociate from this complex and administer branched-out functions. IKKα moves into the nucleus and phosphorylates its nuclear substrates to regulate gene transcription; IKKβ, which could be free from the IKK complex, stays in the cytosol and phosphorylates its cytosolic substrates, such as TSC1, to activate the mTOR pathway and promote protein translation. Thus, IKK complex may regulate the expression of certain genes, such as VEGF, through both transcriptional and translational processes.

Inflammation-Mediated Tumor Angiogenesis

The inflammatory microenvironment in and around tumors contains several types of inflammatory cells, especially tumor-associated macrophages (TAMs), that secrete proinflammatory cytokines and chemokines—including TNFα, IL-1, IL-6, and IL-8—to enhance cell proliferation, cell survival, and angiogenesis and thereby promote tumor

Figure 6. Phosphorylation of TSC1 by IKKβ Impairs the Antiangiogenic Function of TSC1

(A) TSC1(WT) and TSC1(SSAA) profoundly inhibited tumorigenesis, whereas TSC1(SSDD) had little effect. Error bars represent SD (n = 5).

(B-C) The mTOR inhibitor rapamycin inhibited IKKβ-mediated and TSC1(SSDD)-mediated tumorigenesis. Rapamycin (1.5 mg/ kg) was injected into tumor-bearing BALB/c mice three times per week for 2 weeks and then tumor volumes were determined. Symbols are as follows: arrowheads, dates of rapamycin injection; asterisk, date of last treatment. Error bars represent SD (n = 5).

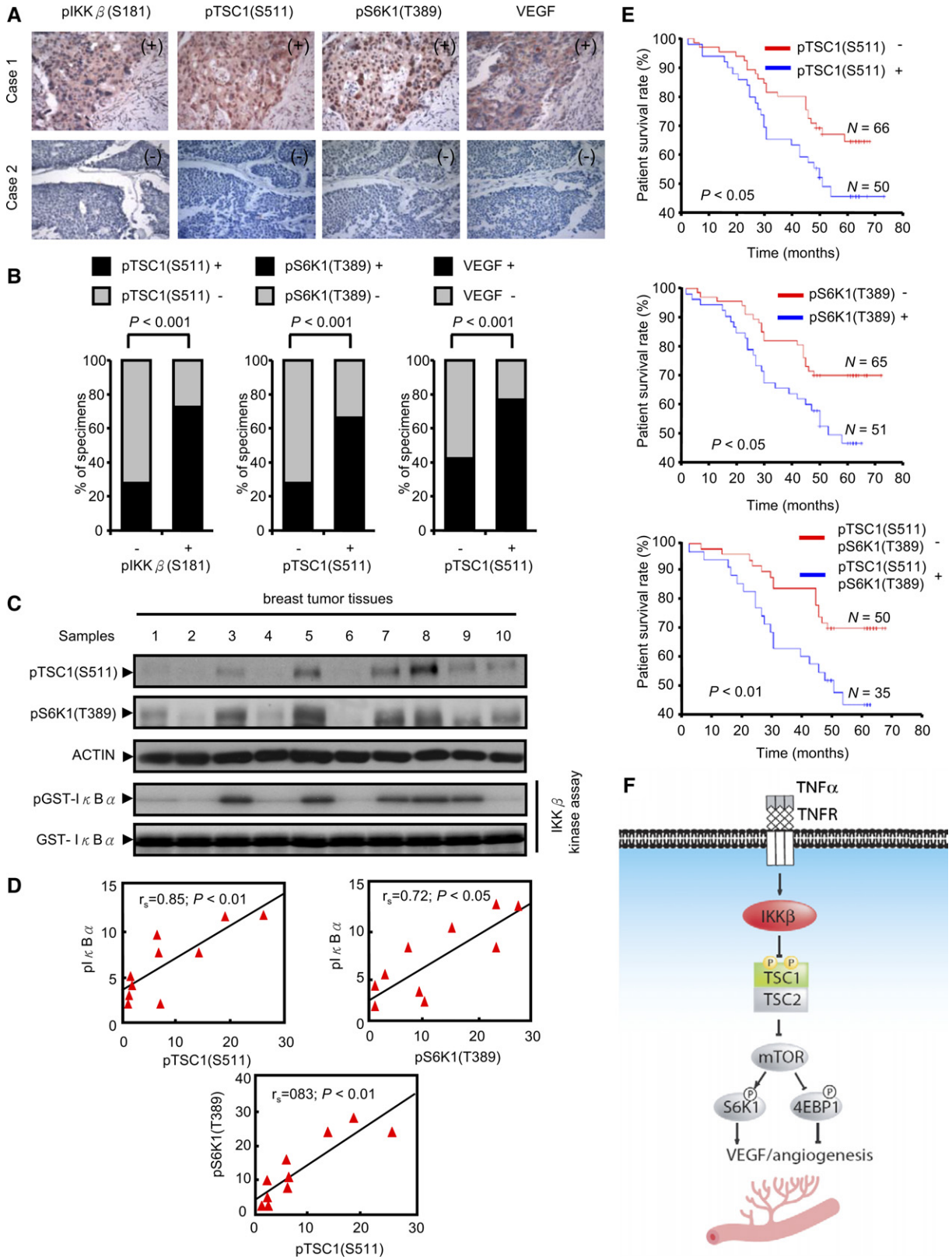
(D) TSC1(SSAA) had a better suppression effect on IKKβ-mediated tumorigenesis than did TSC1(WT). 4T1 mouse mammary tumor cells were cotransfected with various GFP-tagged TSC1 constructs and (n)IKKβ, and GFP-positive cells were sorted by flow cytometry. The sorted cells were then injected into the mammary fat pads of BALB/c mice for in vivo tumorigenesis assays. Error bars represent SD (n = 5).

(E) 4T1-TSC1(SSDD) tumor had greater blood vessel density than did 4T1-TSC1(WT) and 4T1-TSC1(SSAA) tumors in vivo. Frozen tumor sections were fixed, stained with CD31 (red) antibody to permit determination of the number of blood vessels, and counterstained with TOPRO-3 dye (blue) to permit visualization of nuclei. Error bars represent SD (n = 3).

(F) 453-TSC1(SSDD) stable transfectant had a weaker ability to repress HUVEC tube formation than did 453-TSC1(WT) and 453-TSC1(SSAA) stable transfectants. Tube formation assays were performed by culturing HUVECs with the supernatants of various TSC1 stable transfectants. Error bars represent SD (n = 3).

(G) 453-TSC1(SSDD) stable transfectant secreted more VEGF than did 453-TSC1(WT) and 453-TSC1(SSAA) stable transfectants. VEGF secretion from various TSC1 stable transfectants was quantified by ELISA. Error bars represent SD (n = 3).

(H) Depletion of VEGF by antibody to VEGF (α-VEGF) repressed HUVEC tube formation in various TSC1 stable transfectants. Error bars represent SD (n = 3).


A
pIKK β (S181)
pTSC1(S511)
pS6K1(T389)
VEGF
Case 1
Case 2
(+)
(-)
B
pTSC1(S511) +
pTSC1(S511) -
pS6K1(T389) +
pS6K1(T389) -
VEGF +
VEGF -
P < 0.001
% of specimens
pIKK β (S181)
pTSC1(S511)
C
breast tumor tissues
Samples 1 2 3 4 5 6 7 8 9 10
pTSC1(S511)
pS6K1(T389)
ACTIN
pGST-I κ B α
GST- I κ B α
IKK β kinase assay
D
r_s=0.85; P < 0.01
r_s=0.72; P < 0.05
r_s=083; P < 0.01
pI κ B α
pTSC1(S511)
pS6K1(T389)
E
Patient survival rate (%)
Time (months)
pTSC1(S511) -
pTSC1(S511) +
N = 66
N = 50
P < 0.05
pS6K1(T389) -
pS6K1(T389) +
N = 65
N = 51
P < 0.05
pTSC1(S511) pS6K1(T389) -
pTSC1(S511) pS6K1(T389) +
N = 50
N = 35
P < 0.01
F
TNFα
TNFR
IKKβ
TSC1
TSC2
mTOR
S6K1
4EBP1
VEGF/angiogenesis

progression. TAMs, derived from circulating monocytic precursors, are widespread in human breast cancers and have been suggested to play a vital role in tumor angiogenesis (Bingle et al., 2002). It has been suggested that the proinflammatory cytokine TNFα induces tumor cells to secrete VEGF through NF-κB-mediated transcriptions of VEGF and hypoxia-inducible factor-1β. Recently, TBK1, an IKK kinase involved in both NF-κB and IRF3 signaling (Fitzgerald et al., 2003), was identified as a proangiogenic trigger by high-throughput functional genomic screen (Korherr et al., 2006), implying that inflammatory signaling pathways play important roles in promoting tumor angiogenesis. Our finding that TNFα mediates tumor angiogenesis through dysregulated mTOR signaling caused by suppression of TSC1 by IKKβ reveals a novel mechanism of inflammation-mediated tumor angiogenesis. Collectively, these findings suggest that inflammation-mediated tumor angiogenesis may occur through both transcriptional (NF-κB and IRF3) and translational controls (mTOR) by multiple distinct inflammation-associated kinases, including IKKβ and TBK1.

The identification of TSC1 as a downstream effector of IKKβ links the TNFα and mTOR signaling pathways and provides an important new starting point for uncovering the molecular basis of TNFα-mediated human tumor angiogenesis and identifying potential targets for antiangiogenic therapy. Further investigations of the IKKβ/TSC1/mTOR signaling pathway may identify new molecules involved in regulating TNFα-induced pathological disorders. Preventing the phosphorylation of TSC1 may have important clinical implications for the treatment or prevention of cancer.

EXPERIMENTAL PROCEDURES

Supplemental Experimental Procedures are provided in the Supplemental Data.

Cell Culture, Stable Transfectants, and Transfection

MCF-7, MDA-MB-453, HBL-100, RAW264.7, and HEK293 cells and *Ikkβ*$^{-/-}$, *Tsc1*$^{+/+}$, *Tsc1*$^{-/-}$, *Tsc2*$^{+/+}$*p53*$^{-/-}$, and *Tsc2*$^{-/-}$*p53*$^{-/-}$ MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal bovine serum. MCF-10A was cultured in DMEM/F12 medium supplemented with 5% horse serum, 10 μg/ml insulin, 20 ng/ml EGF, 100 ng/ml cholera toxin, and 500 ng/ml hydrocortisone. IKKβ and TSC1 stable transfectants were selected by blasticidin S and G418, respectively. For transient transfection, cells were transfected with DNA by either SN liposome (Zou et al., 2002), lipofectamine with plus reagent, or electroporation using a Nucleofector 1 device (Amaxa) with electroporation buffer (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, and 20 mM HEPES, pH 7.0). To investigate the effects of agonists on pS6K1(T389), the transfected cells were serum-starved overnight and extracted directly or after stimulation with TNFα, IL-1β, LPS, IGF1, EGF, WNT3a, TGF-β1, glucose, or amino acids for 30 min.

Subcellular Fractionation

Membrane-bound and cytosolic TSC1 and TSC2 proteins were separated by hypotonic buffer as previously described (Cai et al., 2006).

Mouse Model for Tumorigenesis and Angiogenesis Studies

The tumorigenesis assays were performed using an orthotopic breast cancer mouse model as previously described (Chang et al., 1997). The mouse mammary tumor cell line 4T1 was transfected with various GFP-tagged TSC1 constructs, and the GFP-positive cells were sorted by flow cytometry. Cells (1×10^5) were injected into the mammary fat pads of BALB/c mice (The Jackson Laboratory; 5 mice per group), and the volumes of the resulting tumors were measured twice a week after tumors were detected by an IVIS imagining system (Xenogen). Angiogenesis was examined by CD31 immunostaining. The data were analyzed using Student's t test. For intratumoral injection, the 4T1 cells were inoculated in the mammary fat pads of the mice. When tumor size reached about 7 mm^3, various TSC1 constructs (50 μg) complexed with DOTAP:Chol liposome (50 μl) were injected into tumors three times per week. After 1 week of treatment, mice were sacrificed, and tumor samples were examined for angiogenesis by CD31 immunostaining.

Immunohistochemical Staining and Human Breast Tumor Samples

Immunohistochemical staining was performed as previously described (Deng et al., 2002; Hu et al., 2004). Human HistoArrays IMH-365 (multiple cancerous tissues), IMH-301 (normal organs), and IMH-337 (normal tissues) were purchased from Imgenex. Human tissue specimens were incubated with antibodies to pTSC1(S511), pS6K1(T389), pIKKβ(S181), VEGF, or TNFα and biotin-conjugated secondary antibody and then incubated with avidin-biotin-peroxidase complex, and visualization was performed with amino-ethylcarbazole chromogen. The human breast tumor samples for IKKβ kinase assay and Western blotting were received from the breast tumor bank of The University of Texas M. D. Anderson Cancer Center. Fisher's exact test and Spearman rank correlation were used for statistical analysis; $p < 0.05$ was considered statistically significant. According to histologic scoring, the intensity of staining was ranked into four groups: high (score 3), medium (score 2), low (score 1), and negative (score 0). The immunoreactivities for pTSC1(S511), pS6K1(T389), pIKKβ(S181), VEGF, and TNFα were semiquantitatively scored using a well-established immunoreactivity score system in which immunoreactivity

Figure 7. Clinical Association between pTSC1(S511) Expression; pIKKβ(S181), pS6K1(T389), and VEGF Expression, and Survival of Breast Cancer Patients

(A) pTSC1(S511) level was associated with pIKKβ(S181), pS6K1(T389), or VEGF expression in 115 primary human breast cancer specimens. Shown are two representative specimens.

(B) Left panel shows percentages of specimens with low (–) or high (+) pIKKβ(S181) expression in which pTSC1(S511) was or was not observed. Middle and right panels show the percentages of specimens with low (–) or high (+) pTSC1(S511) expression in which pS6K1(T389) or VEGF expression was or was not observed.

(C) IKKβ activity was associated with pTSC1(S511) and pS6K1(T389) expression in 10 freshly prepared human breast tumor lysates. IKKβ activity was determined by in vitro kinase assay, and the expression levels of pTSC1(S511) and pS6K1(T389) were determined by immunoblotting.

(D) Correlation analyses between IKKβ kinase activity and pTSC1(S511) and pS6K1(T389) expression levels were performed by Spearman rank correlation.

(E) The Kaplan-Meier overall survival curves indicated that pTSC1(S511) and pS6K1(T389), alone and together, are associated with a reduction of overall survival in breast cancer patients.

(F) A model in which phosphorylation of TSC1 by IKKβ activates the mTOR pathway and thereby induces tumor angiogenesis.

score was generated by incorporating both the percentage of positive tumor cells and the intensity of staining (Camp et al., 1999). An immunoreactivity score less than 150 was considered negative (−); an immunoreactivity score greater than 150 was considered positive (+).

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, three tables, and twelve figures and can be found with this article online at http://www.cell.com/cgi/content/full/130/3/440/DC1/.

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