Regulation of tumor angiogenesis by integrin-linked kinase (ILK)

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Summary

We show that integrin-linked kinase (ILK) stimulates the expression of VEGF by stimulating HIF-1α protein expression in a PKB/Akt- and mTOR/FRAP-dependent manner. In human prostate cancer cells, knockdown of ILK expression with siRNA, or inhibition of ILK activity, results in significant inhibition of HIF-1α and VEGF expression. In endothelial cells, VEGF stimulates ILK activity, and inhibition of ILK expression or activity results in the inhibition of VEGF-mediated endothelial cell migration, capillary formation in vitro, and angiogenesis in vivo. Inhibition of ILK activity also inhibits prostate tumor angiogenesis and suppresses tumor growth. These data demonstrate an important and essential role of ILK in two key aspects of tumor angiogenesis: VEGF expression by tumor cells and VEGF-stimulated blood vessel formation.

Introduction

Angiogenesis plays a critical role in cancer progression (Hanahan and Weinberg, 2000). Tumor growth and metastasis have been shown to be dependent on angiogenesis, and inhibition of tumor angiogenesis by selectively inhibiting the growth, survival, and migration of endothelial cells is perceived as an attractive, nontoxic means of regulating tumor progression (Kerbel, 1991).

A variety of proteins have been identified as potential targets of antiangiogenesis therapy, and despite poor results in clinical trials of some of the antiangiogenic strategies (Kerbel and Folkman, 2002), the potential of antiangiogenic therapy continues to be an attractive means of cancer control.

One of the key mediators of angiogenesis is vascular endothelial cell growth factor (VEGF), which can promote the proliferation, survival, and migration of endothelial cells and is essential for blood vessel formation (Ferrara, 2002). VEGF is expressed by activated endothelial cells, but more importantly for tumor angiogenesis, VEGF expression and secretion are stimulated in tumor cells by activation of oncogenes such as Ras (Rak et al., 2000), as well as by the activation of the PI-3 kinase pathway (Jiang et al., 2001; Fukuda et al., 2002), which has inherent oncogenic properties. The PI-3 kinase pathway can be constitutively activated via autocrine growth factors by constitutively activated growth factor receptors such as Erb-B2, by activating mutations in PI-3 kinase or its downstream effector, PKB/Akt, or by the mutational inactivation, or loss of the tumor suppressor, PTEN (Cantley and Neel, 1999). The constitutive upregulation of expression of VEGF by tumor cells is felt to be a major contributor to tumor angiogenesis (Ferrara, 2002).

VEGF expression is regulated at the level of transcription by a variety of transcription factors that include AP-1, NF-κB, and hypoxia-inducible factor-1α (HIF-1α) (Huang et al., 2000; Ryan et al., 1998; Damert et al., 1997). The major physiological stimulus for VEGF expression is hypoxia, resulting in the transcriptional induction of the VEGF gene by HIF-1α (Forsythe et al., 1996; Carmeliet et al., 1998; Ryan et al., 1998), which is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits (Jiang et al., 1996). The hypoxia-mediated stimulation of HIF-1α expression is regulated by the inhibition of ubiquitin-mediated degradation and consequent stabilization of the HIF-1α subunit under hypoxic conditions. As a result, HIF-1α accumulates, dimerizes with HIF-1β, and activates transcription of target genes, including VEGF (reviewed in Harris, 2002). Recently, however, the expression of VEGF via the activation of the PI-3 kinase pathway has also been shown to be mediated by HIF-1α (Jiang et al., 2001; Fukuda et al., 2002). Signaling via receptor tyrosine kinases induces HIF-1α expression by an independent mechanism involving the stimulation of increased rates of HIF-1α protein synthesis via PI-3 kinase-dependent

SIGNIFICANCE

Constitutive activation of the PI-3 kinase pathway is oncogenic and is implicated in the promotion of tumor angiogenesis by stimulating the expression of VEGF. The integrin-linked kinase (ILK) is a PI-3 kinase-dependent effector of integrin-mediated cell adhesion as well as growth factors and is an upstream regulator of PKB/Akt. Here we show that ILK is essential for HIF-1α and VEGF expression in prostate cancer cells and that it is also essential for VEGF-stimulated endothelial cell migration, tube formation, and tumor angiogenesis. Consequently, ILK plays important roles in two key aspects of tumor angiogenesis: VEGF expression by tumor cells and VEGF-stimulated blood vessel formation. Our findings suggest that ILK is a promising therapeutic target for the inhibition of tumor angiogenesis.

stimulation of PKB/Akt and mTOR/FRAP, which activates the translational regulatory protein eIF-4E binding protein 1 (4E-BP1) and p70 S6 kinase (Fukuda et al., 2002; Laughner et al., 2001; Gingras et al., 2001; Peterson et al., 1999). These findings indicate that HIF-1α regulates both hypoxia- and growth factor-induced VEGF expression.

One of the components of the PI-3 kinase pathway, immediately upstream of PKB/Akt, is integrin-linked kinase (ILK) (Hannigan et al., 1996; Wu and Dedhar, 2001; Troussard et al., 2003). ILK can interact with the cytoplasmic domain of β-integrin subunits and is activated by both integrin activation as well as growth factors (Wu and Dedhar, 2001). ILK is a PI-3 kinase-dependent kinase (Persad et al., 2001a; Delcommenne et al., 1998) and is an upstream regulator of the phosphorylation of PKB/Akt on serine 473 (Troussard et al., 2003; Persad et al., 2000, 2001a; Lynch et al., 1999; Delcommenne et al., 1998), one of the two phosphorylation sites required for the full activation of PKB/Akt. Overexpression of ILK induces anchorage-independent cell growth and suppression of anoikis and promotes hyperplasia and tumor formation in vivo (Wu and Dedhar, 2001). ILK activity is also constitutively activated in PTEN null cancer cells, and the constitutive activation of PKB/Akt in such cells is inhibited upon inhibition of ILK activity (Persad et al., 2000). ILK also promotes cell migration and invasion (Persad and Dedhar, 2003). Because of these oncogenic properties of ILK, we decided to explore the potential role of ILK in promoting tumor angiogenesis. We wanted to determine whether ILK is involved in the stimulation of expression of VEGF in tumor cells and secondly whether ILK is required for VEGF-mediated endothelial cell migration and formation of blood vessels.

In this paper, we report that overexpression of ILK stimulates VEGF expression in a PKB/Akt- and HIF-1α-dependent manner and that inhibition of ILK expression or activity in VEGF-expressing prostate cancer cells (DU145 and PC3) results in dramatic inhibition of VEGF expression and secretion via inhibition of PKB/Akt activity and HIF-1α expression. Furthermore, inhibition of ILK activity or expression in VEGF-stimulated endothelial cells results in the inhibition of endothelial cell migration and blood vessel formation in vitro and in vivo. A small molecule ILK inhibitor suppresses tumor angiogenesis and tumor growth in a PC3 xenograft tumor model. Our results demonstrate an important and essential role of ILK in two key aspects of tumor angiogenesis, VEGF expression by tumor cells and VEGF-stimulated blood vessel formation, and suggest that ILK may be a promising therapeutic target for the inhibition of tumor angiogenesis.

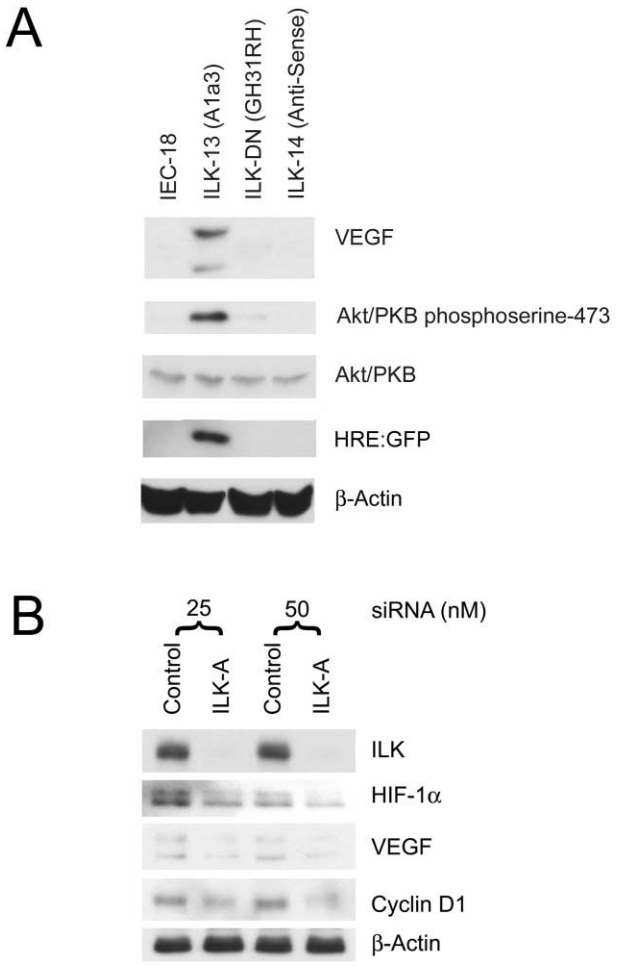

Figure 1. VEGF expression and HIF-1α activity are increased in epithelial cells with a high ILK activity

A: Immunoblot analysis with the indicated antibodies of NP-40 cell lysates of the indicated cell lines transfected with 3 μg HRE:GFP and pRenilla (transfection efficiency control) and exposed (24 hr) to 1% FBS media.
B: Immunoblot analysis with the indicated antibodies of the RIPA IEC-18 ILK 13 A1a3 cell lysates 4 days posttransfection with the indicated type and amount of siRNA. All figures are a representation of three trials.

Results

Overexpression of ILK stimulates VEGF expression in a PKB/Akt- and HIF-1α-dependent manner

We have previously demonstrated that overexpression of ILK in IEC-18 rat intestinal epithelial cells results in anchorage-independent cell cycle progression, tumorigenicity in nude mice, activation of PKB/Akt, inhibition of GSK-3, and stimulation of AP-1, NF-κB, and β-catenin/LEF transcription factors (reviewed in Wu and Dedhar, 2001). Overexpression of a kinase-deficient mutant of ILK, or ILK antisense cDNA, did not result in the stimulation of these pathways or phenotypes. Because activation of other oncogenes such as Ras or the PI-3 kinase pathway has been shown to stimulate VEGF expression in tumor cells (Rak et al., 2000), we wanted to determine whether ILK overexpression also resulted in the stimulation of VEGF expression. As shown in Figure 1A, the expression of both isoforms of VEGF is markedly stimulated in the ILK-overexpressing clone of IEC-18 cells (ILK-13, A1a3), as compared to control clones expressing the E359K kinase-deficient ILK dominant-negative (ILK-DN, GH31RH) or antisense-ILK (ILK-14) (Novak et al., 1998; Hannigan et al., 1996). In addition, the data in Figure 1A also show markedly increased phosphorylation of PKB/Akt on serine 473 in the absence of any changes in PKB/Akt expression. Since one of the major transcriptional regulators of the VEGF gene is hypoxia-inducible factor-1α (HIF-1α), we transfected the different IEC-18 clones described above with a HIF-1 response element fused to a green fluorescence protein (GFP) reporter (HRE:GFP) (Ruan and Deen, 2001). As shown in Figure 1A, this reporter is only active in the ILK-overexpressing clone, suggesting that the stimulation of VEGF expression in these cells is likely mediated by the upregulation or activation of the HIF-1

transcription factor. We were unable to directly analyze HIF-1α protein expression in these clones because of the lack of availability of suitable anti-rat HIF-1α antibodies. Inhibition of ILK expression in the A1a3 ILK overexpressing cells with ILK siRNA resulted in the suppression of VEGF expression (Figure 1B), showing that ILK is indeed responsible for the stimulation of VEGF expression in these cells. We have previously shown that the ILK-overexpressing clones have constitutive high-level expression of cyclin D1 (Radeva et al., 1997), and inhibition of ILK expression by siRNA inhibits cyclin D1 expression (Troussard et al., 2003). As shown in Figure 1B, ILK siRNA also results in the suppression of cyclin D1 expression in the ILK-overexpressing cells. These data demonstrate that overexpression of kinase-active ILK results in the stimulation of VEGF expression via the activation of PKB/Akt and the HIF-1 transcription factor.

Inhibition of ILK expression and activation suppresses PKB/Akt and mTOR/FRAP phosphorylation and inhibits HIF-1α and VEGF expression in prostate cancer cells

In order to analyze in more detail the ILK-mediated signaling pathway leading to the stimulation of VEGF and to assess the relevance of ILK in VEGF expression in cancer cells, we decided to inactivate ILK expression or activity in human prostate cancer cells that express VEGF.

The PI-3 kinase pathway is constitutively activated in many cancer cells lines. In certain human prostate cancer cell lines (PC3 and LnCAP), the PI-3 kinase pathway is constitutively activated due to the loss of expression of the tumor suppressor PTEN (Davies et al., 1999; Stambolic et al., 1998). We have previously shown that ILK activity is also constitutively activated in these cells and that inhibition of ILK activity suppresses PKB/Akt activity in these PTEN null cells (Persad et al., 2000). VEGF expression has been shown to be constitutively elevated in PC3 cells (Jiang et al., 2001). Because we had found that ILK stimulated VEGF expression (Figure 1), we wanted to determine whether inhibition of ILK activity in PC3 cells resulted in the inhibition of VEGF expression. As shown in Figure 2A, inhibition of ILK activity by transfection of kinase-deficient, ILK-DN (E359K) (Persad et al., 2000), or wild-type PTEN resulted in inhibition of VEGF expression at the protein level as determined by Western blotting. In addition, the expression of HIF-1α protein is also substantially inhibited by DN-ILK and PTEN (Figure 2A). As expected, DN-ILK also inhibited phosphorylation of PKB/Akt on serine 473 (Figure 2A). Expression of dominant-negative ILK and PTEN also inhibited the activity of the HIF-1 response element (HRE), as shown in Figure 2C, suggesting that the upregulation of VEGF expression in these cells is likely due to the ILK-mediated upregulation of HIF-1α expression.

We have recently utilized double-stranded RNA interference (siRNA) to knock down ILK protein expression (Troussard et al., 2003). Furthermore, we have shown that ILK knockdown by siRNA results in significant inhibition of PKB/Akt serine 473 phosphorylation and activation (Troussard et al., 2003). We therefore exposed PC3 cells to increasing concentrations of ILK-specific siRNA (Troussard et al., 2003). As shown in Figure 2B, ILK siRNA resulted in the complete depletion of ILK expression in PC3 cells. This was associated with a suppression of phosphorylation of PKB/Akt on serine 473. Expression of PKB/Akt was not affected (Figure 2B). Furthermore, ILK siRNA-mediated knockdown of ILK also resulted in significant inhibition of expression of both HIF-1α and VEGF protein (Figure 2B). It has

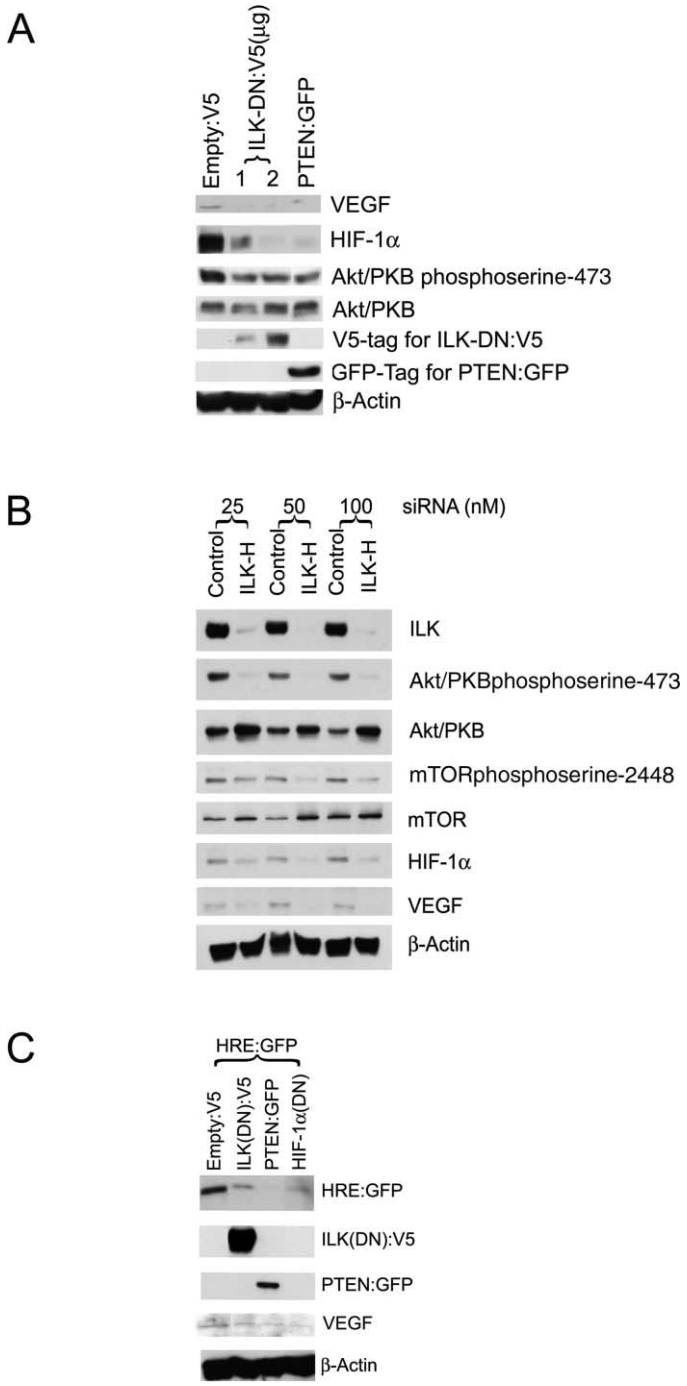

Figure 2. VEGF and HIF-1α expression are severely affected by the loss of ILK activity in a PTEN- and mTOR/FRAP-dependent manner in PTEN null prostate carcinoma cells (PC3)

A: Immunoblot analysis with the indicated antibodies of the RIPA lysate of PC3 cells transfected with 1–2 μg of Empty:V5, ILK-DN:V5 or PTEN:GFP vector.
B: Immunoblot analysis with the indicated antibodies of the RIPA lysate of PC3 cells transfected with the indicated type and amount of siRNA.
C: Immunoblot analysis with the indicated antibodies of NP-40 lysate of PC3 cells cotransfected with the indicated constructs and pRenilla after 48 hr. All figures are a representation of three trials.

been recently shown that PKB/Akt can regulate the expression of HIF-1α protein at the translational level by stimulating the phosphorylation of mTOR/FRAP, which is a regulator of protein synthesis (Gingras et al., 2001; Peterson et al., 1999). We therefore wanted to determine whether the ILK-mediated expression of HIF-1α and VEGF also involved mTOR/FRAP. As shown in Figure 2B, siRNA-mediated knockdown of ILK resulted in the inhibition of mTOR/FRAP phosphorylation on serine 2448, concomitant with the inhibition of the PKB/Akt phosphorylation. The expression of mTOR/FRAP protein was not affected by the knockdown of ILK. These data suggest that in the PC3 cells, the constitutive activation of ILK drives VEGF expression most likely via HIF-1α through the activation of PKB/Akt and mTOR/FRAP, resulting in increased translation of HIF-1α protein. This is further substantiated by the observation that transfection of a dominant-negative HIF-1α construct into PC3 cells almost completely inhibits VEGF expression as well as HRE activity (Figure 2C).

Pharmacological inhibition of ILK activity results in the inhibition of HIF-1α and VEGF expression in prostate cancer cells

We have identified highly selective small molecule inhibitors of ILK activity. These ATP competitive inhibitors have been extensively characterized, and shown to inhibit ILK activity and the activation of all of the downstream effectors of ILK (Cruet-Hennequart et al., 2003; Mills et al., 2003; Persad et al., 2000, 2001a, 2001b; Tan et al., 2001, 2002; Troussard et al., 2000). The inhibitors are equally effective and specific as ILK inhibition by dominant-negative ILK and ILK siRNA (Persad et al., 2001a, 2001b; Troussard et al., 2003). We therefore wanted to determine whether exposure of human prostate cancer cells to the ILK inhibitor would also inhibit the expression of HIF-1α and the expression and synthesis of VEGF. As shown in Figure 3A, exposure of both PC3 and DU145 prostate cancer cells to the ILK inhibitor KP-392 (Persad et al., 2001a, 2001b) resulted in the inhibition of both HIF-1α and VEGF expression in a dose-dependent manner. Despite poor cellular permeability of this inhibitor, resulting in the exposure of cells to relatively high concentrations, it can be seen that there is significant inhibition of both HIF-1α and VEGF expression at 25 μM KP-392, especially in PC3 cells As well, it can clearly be seen that KP-392 also suppresses the phosphorylation of PKB/Akt on serine 473 in a dose-dependent manner. Again, there is no effect on the expression of PKB/Akt. In addition, as shown in Figure 3B, not only VEGF cellular expression but also its secretion, as determined by an enzyme-linked immunosorbant assay (ELISA) of the conditioned cell media, are inhibited by the ILK inhibitor KP-392. In contrast to PC3 cells, VEGF expression is not completely inhibited by the ILK inhibitor in DU145 cells, despite substantial inhibition of HIF-1α expression. This suggests cell type differences in the regulation of VEGF expression.

In Figure 3C, we demonstrate that HIF-1α expression is stimulated by serum in serum-starved PC3 cells and that inhibition of ILK as well as PI-3 kinase with the respective pharmacological inhibitors KP-392 and LY294002 inhibits HIF-1α expression.

Collectively, the data shown in Figures 2 and 3 demonstrate that ILK is a critical component of the constitutively activated PI-3 kinase-PKB/Akt signaling pathway resulting in the stimulation of HIF-1α and VEGF. Inhibition of ILK expression or activity

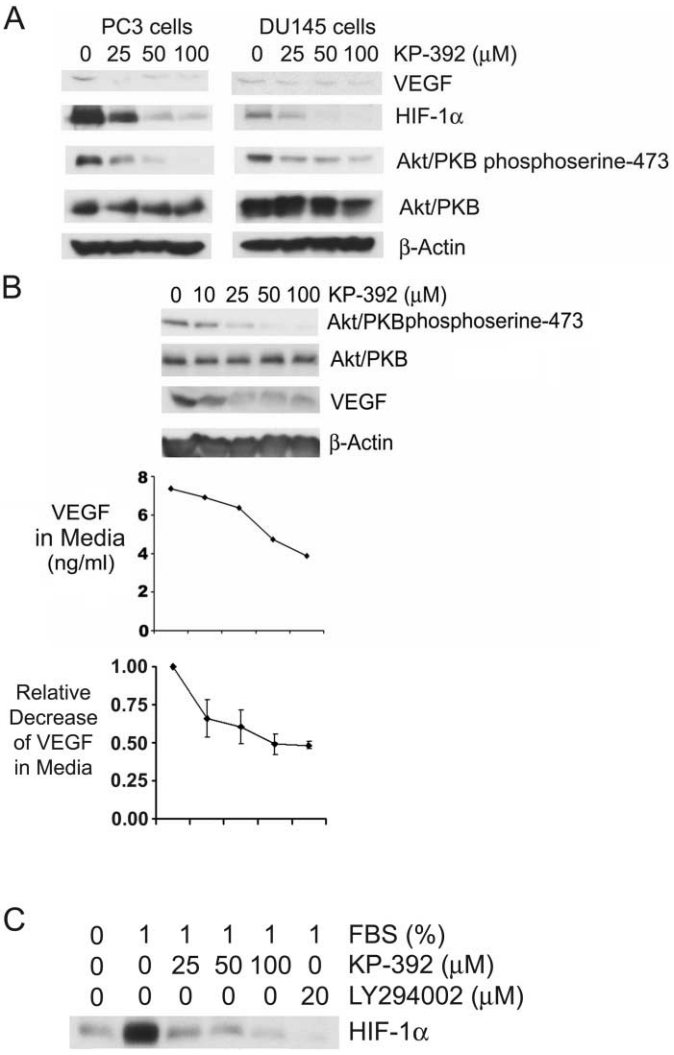

Figure 3. Inhibition of ILK activity results in decrease of HIF-1α and VEGF expression in a PI-3 kinase-dependent manner in prostate carcinoma cells

A: Immunoblot analysis with the indicated antibodies of RIPA lysate of PC3 and DU145 cells starved (20 hr) and then exposed (24 hr) to the indicated amounts of KP-392, equivalent amounts of DMSO vehicle, and 1% FBS.
B: Quantification by ELISA of the level of secreted VEGF protein in conditioned media.
C: Immunoblot analysis of RIPA lysates of DU145 cells cultured under the indicated conditions (24 hr). All figures are a representation of three trials.

can result in substantial inhibition of the expression of both HIF-1α and VEGF, suggesting that ILK may be an important therapeutic target for the inhibition of expression of the angiogenic factor, VEGF.

ILK regulates VEGF-mediated endothelial cell migration and blood vessel formation

VEGF stimulates endothelial cell survival and migration and promotes the formation of new blood vessels (Ferrara, 2002). Since the activity of ILK is stimulated by various growth factors and chemokines (Wu and Dedhar, 2001; Friedrich et al., 2002) and ILK also promotes cell migration and invasion (Persad and Dedhar, 2003), we wanted to determine whether ILK also played a role in VEGF-mediated endothelial cell migration and vascular

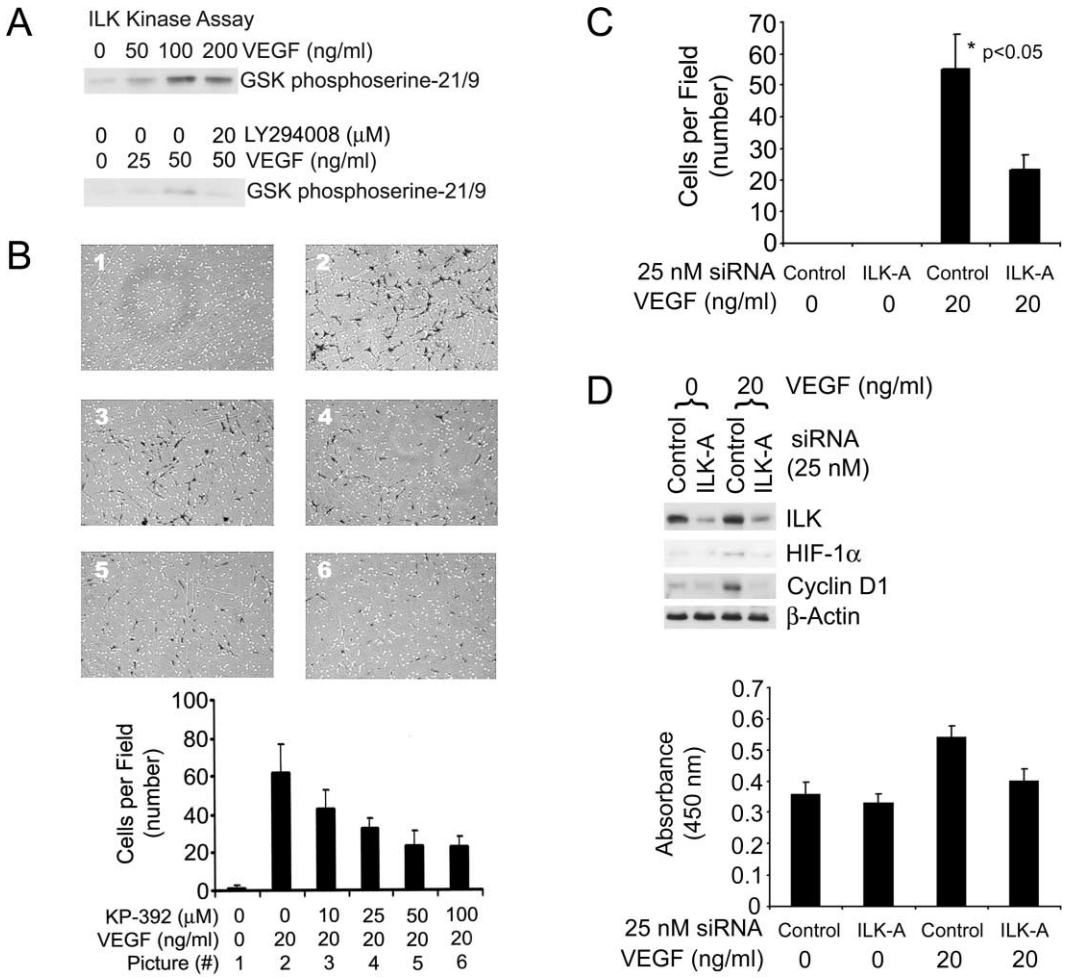

Figure 4. ILK kinase activity is involved in VEGF-stimulated HUVEC activity

A: ILK kinase activity is stimulated by VEGF in HUVEC. Cells were starved (24 hr) and exposed to the indicated amounts of VEGF and LY294002. The ILK kinase assay was carried out as described in the Experimental Procedures. This is a representation of three independent trials.

B: Decrease in ILK activity reduces HUVEC invasion and migration toward VEGF. Two hours after HUVEC were seeded on the upper chamber, indicated amounts of KP-392 were added to this chamber, and the migration assay was performed and analyzed as described in Experimental Procedures. This graph represents the mean of three experiments ± SD.

C: Knockdown of ILK expression reduces HUVEC invasion and migration toward VEGF. Equal number of HUVEC transfected for 3 days with the indicated siRNA (25 nM) were seeded in the upper chamber. The experiment was performed as described in Experimental Procedures. This graph represents the mean of three experiments ± SD.

D: Immunoblot analysis with the indicated antibodies of RIPA lysate of transfected HUVEC (25 nM indicated siRNA) that were starved (24 hr) then exposed (24 hr) to VEGF (0 or 20 ng/ml) 2 days posttransfection. The graph represents relative HUVEC growth after above conditions, measured by WST-1/ECS assay. Results represent mean absorbance ± SD. This is a representation of three experiments.

morphogenesis. As shown in Figure 4A, VEGF stimulates ILK kinase activity in a dose-dependent manner in quiescent human umbilical vein endothelial cells (HUVEC). The stimulation of ILK activity by VEGF is dependent on PI-3 kinase activity since the VEGF stimulation of ILK activity is inhibited in the presence of the PI-3 kinase inhibitor, LY294002. These data support previous studies showing ILK to be a PI-3 kinase-dependent kinase (Delcommenne et al., 1998; Lynch et al., 1999) and demonstrate that VEGF stimulates ILK activity in a PI-3 kinase-dependent manner. We next determined whether ILK was required for the stimulation of VEGF-mediated cell migration of HUVEC cells. As shown in Figure 4B, VEGF stimulates the migration of HUVEC cells, and inhibition of ILK activity with the pharmacological ILK inhibitor, KP-392, results in a dose-dependent inhibition of VEGF-mediated HUVEC cell migration. Furthermore, inhibition of ILK expression in HUVEC cells by ILK siRNA also inhibited VEGF-stimulated HUVEC cell migration (Figure 4C), demonstrating an essential role for ILK in the stimulation of endothelial cell migration by VEGF. We also noted inhibition of cyclin D1 expression in the ILK siRNA transfected HUVEC cells (Figure 4D), indicating that inhibiting ILK may also inhibit VEGF-stimulated HUVEC cell proliferation. As shown in Figure 4D, HUVEC cell proliferation in response to VEGF is inhibited in the ILK siRNA transfected cells. Cell viability in the ILK siRNA transfected cells was not significantly altered (data not shown). These data demonstrate an essential role of ILK in VEGF–mediated HUVEC cell migration and proliferation. It has been demonstrated that VEGF promotes its own expression in endothelial

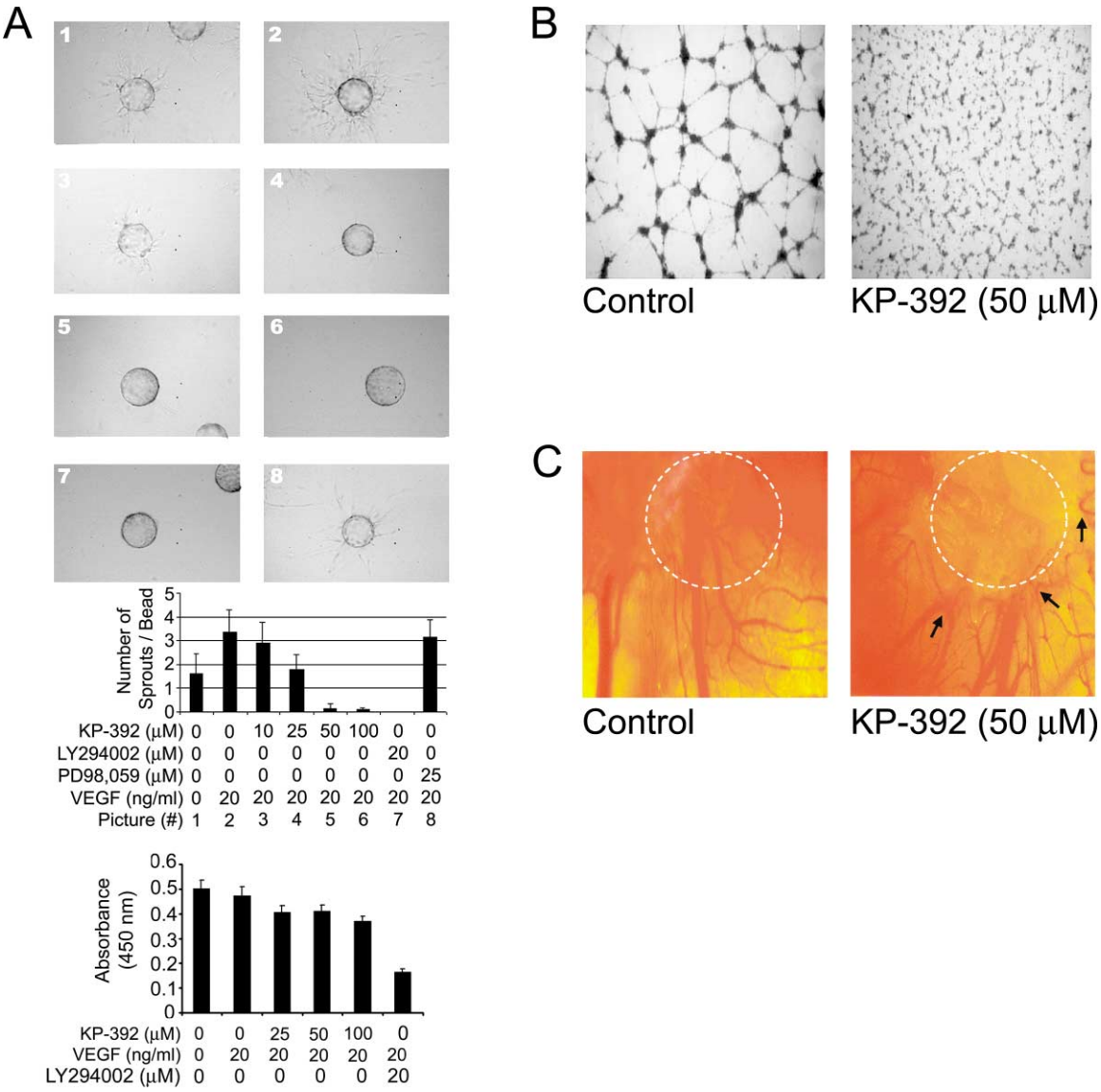

Figure 5. KP-392 inhibits angiogenesis in matrigel and in vivo

A: HUVEC-coated beads embedded in fibrin were incubated in the indicated conditions (VEGF [0 or 20 ng/ml]; KP-392 [μM]). Micrographs of a typical bead were taken at 72 hr. Number of capillary-like tubes formed per microcarrier bead (sprouts/bead) were counted and analyzed as described in Experimental Procedures. The graph represents the mean of three experiments ± SD. The graph below represents relative cell viability after exposure (24 hr) to the indicated amounts of KP-392, LY294002, and VEGF, measured by WST-1/ECS assay. Results represent mean absorbance ± SD.

B: HUVEC suspension in Matrigel were incubated with VEGF and DMSO (Control) or 50 μM KP-392. Micrographs of a typical field were taken to illustrate HUVEC tube formation.

C: Photographs of developing chick chorioallantoic membranes incubated with 30 ng/ml VEGF and DMSO (Control) or 50 μM KP-392. Dashed circle outlines the area covered by the gelatin sponges. Arrows show blood vessels migrating away from the area containing KP-392. All figures are a representation of three independent experiments.

cells via a positive autocrine loop involving HIF-1α expression and activity (Stoeltzing et al., 2003; Zhong et al., 2000). It is therefore interesting to note that VEGF-stimulated HIF-1α expression in HUVEC cells is inhibited by ILK siRNA (Figure 4D), suggesting that ILK is a component of this positive feedback loop.

We next wanted to determine whether ILK is required for VEGF-mediated blood vessel formation. To evaluate this, we initially utilized an in vitro endothelial cell sprouting assay. As shown in Figure 5A, VEGF significantly stimulated HUVEC capillary sprouting, which was quantified as described in the Experimental Procedures. Both the KP-392 ILK inhibitor and the PI-3 kinase inhibitor LY294002 inhibited cell sprouting. Exposure of cells to 50 μM KP-392 and 20 μM LY294002 completely inhibited VEGF-induced HUVEC sprouting. In contrast, the MEK inhibitor, PD98059, did not have any significant inhibitory effect in this assay. The ILK inhibitor had only minor effects on HUVEC cell viability, and only at very high concentrations (Figure 5A). These data demonstrate that PI-3 kinase and ILK activities are required for VEGF-mediated vascular morphogenesis in vitro.

Another assay that is frequently used for the demonstration

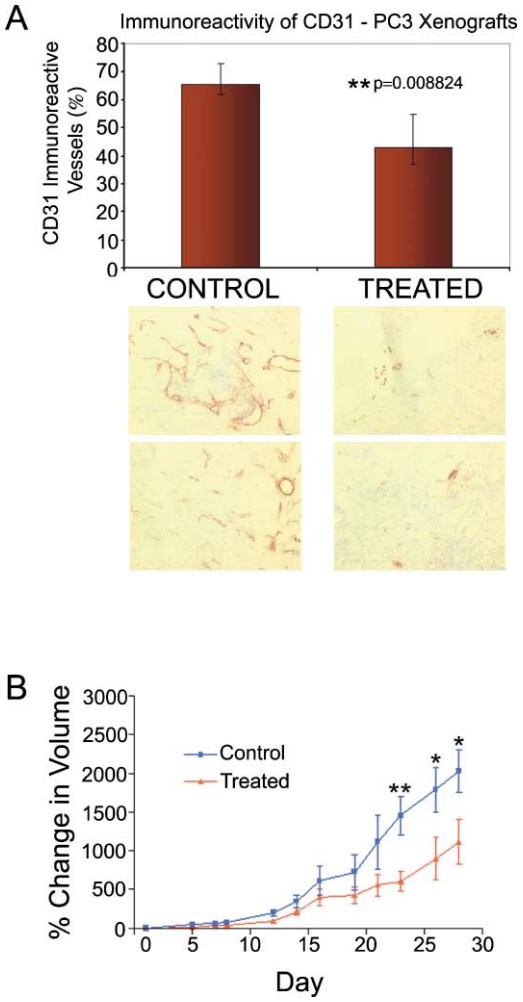

Figure 6. Inhibition of ILK activity suppresses tumor angiogenesis and tumor growth rate

Nude mice with PC3 flank tumors were treated with daily i.p. injection of 100 mg/kg ILK inhibitor or vehicle for 28 days.

A: 7 days after dosing, one tumor from each mouse was harvested and tumor vascular density was analyzed as described in Experimental Procedures. The bar graph shows the means of neovascular densities/field for each group ± SD. Shown are representative photographs of neovasculature in the PC3 tumors.

B: Relative change in tumor volumes in eight mice in the control group and ten mice in the treatment group ± SEM. **: $p < 0.01$; *: $p < 0.05$.

of angiogenesis in vitro is the endothelial tube formation assay in which endothelial cells placed on matrigel in the presence of angiogenic factors results in the endothelial cells forming tube-like structures morphologically similar to capillaries. This tube formation represents the contribution of cell survival, migration, and proliferation (Folkman and Haudenschild, 1980). As shown in Figure 5B, HUVEC cells cultured on matrigel in the presence of VEGF formed tube structures that were completely inhibited by the ILK inhibitor, KP-392. At the same KP-392 concentrations, significant inhibition of HUVEC cell migration and capillary sprout formation were observed (Figures 4B and 5A).

These three different in vitro assays demonstrate that the inhibition of ILK activity has a dramatic effect on endothelial cell function in response to VEGF, suggesting an essential role of ILK in blood vessel formation.

Inhibition of ILK activity inhibits VEGF-stimulated angiogenesis in vivo

We next wanted to determine whether inhibiting ILK activity resulted in the inhibition of VEGF-stimulated angiogenesis in vivo. We utilized a well-established assay for angiogenesis, the chicken chorioallantoic membrane (CAM) assay (Auerbach et al., 1975), to determine the effects of the ILK inhibitor, KP-392. As shown in Figure 5C, inhibition of ILK activity had a significant effect on VEGF-stimulated blood vessel formation in vivo. In the CAM assay (Figure 5C), the incorporation of KP-392, compared to vehicle alone, together with VEGF resulted in the complete blockage of growth of blood vessels toward VEGF. It is interesting to note that the blood vessels are not lysed in the presence of KP-392, but rather they fail to grow toward VEGF and seem to be repelled away from VEGF, demonstrating that the inhibition of ILK predominantly inhibits the migration of endothelial cells and blood vessels toward VEGF.

Inhibition of tumor angiogenesis and suppression of tumor growth in ILK inhibitor-treated PC3 xenograft tumor model

The data presented above suggest that inhibition of ILK activity or expression should inhibit tumor angiogenesis and if PC3 tumor growth in vivo is dependent on tumor vascularization, then ILK inhibition should also induce tumor growth inhibition. To determine whether inhibition of ILK affected tumor angiogenesis and tumor growth in vivo, we established PC3 tumors in nude mice (Figure 6) and treated mice with established tumors with the ILK inhibitor KP-307-2, an analog of KP-392. As shown in Figure 6A, there was a statistically significant effect on tumor vascularization as determined by microvessel density in anti-CD31 stained KP-307-2 treated and control tumor sections. In addition, there was statistically significant tumor growth suppression in the ILK inhibitor-treated mice over a 28 day dosing regimen (Figure 6B). The inhibitor was well tolerated with no obvious side effects or weight loss (data not shown). These data indicate that ILK is a mediator of prostate tumor angiogenesis, and therefore a target for antiangiogenic therapy.

Discussion

Angiogenesis is important in cancer progression and is one of the hallmarks of tumor metastasis (Hanahan and Weinberg, 2000). A principal mediator of tumor angiogenesis is VEGF and a major transcriptional activator of the VEGF gene is HIF-1α (Harris, 2002). It has been reported that the PI-3 kinase/Akt signaling pathway mediates angiogenesis and the expression of VEGF in cells by elevating the levels of HIF-1α protein in cells independent of hypoxic condition (Semenza, 2002). This hypoxia-independent stimulation of HIF-1α and VEGF in cancer cells can be mediated by autocrine or chronic stimulation by growth factors such as IGF-1, constitutive activation of PI-3 kinase, or the constitutive activation of PKB/Akt due to the inactivation of the tumor suppressor, PTEN (Brazil et al., 2002; Galetic et al., 1999). Because ILK is PI-3 kinase dependent and an upstream target of Akt/PKB and because an increase in ILK expression is positively correlated with prostate carcinoma grade (Graff et al., 2001), ILK was a likely candidate to be in-

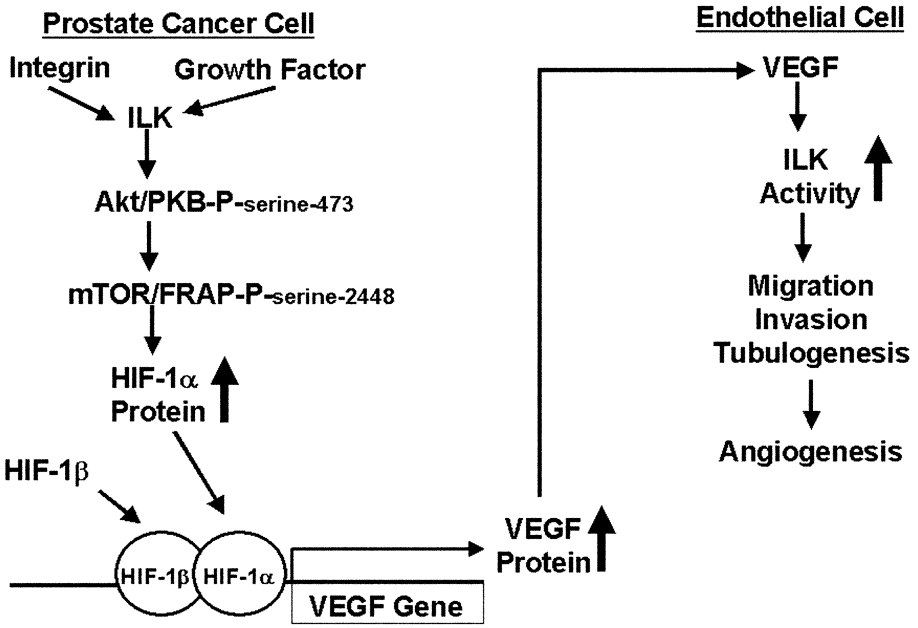

Figure 7. Schematic representation of the cell signaling events leading to VEGF production in a prostate carcinoma cell and consequent effects on a neighboring endothelial cell

Shown is our model for the production of VEGF in prostate carcinoma cell and the effects of VEGF on endothelial cell function. Phosphorylation of serine 473 of Akt/PKB by activated ILK in prostate cancer cells results in the full activation of PKB/Akt that promotes the phosphorylation of serine 2448 of mTOR/FRAP. This activates mTOR/FRAP, which increases the levels of HIF-1α protein translation. HIF-1α protein combines with HIF-1β to form an active transcription factor. This heterodimer binds to the VEGF promoter and activates VEGF transcription, expression, and secretion. VEGF binds to its receptor on nearby endothelial cells and stimulates ILK activity. ILK regulates downstream targets involved in cell survival, proliferation, invasion migration, and sprout formation, which result in angiogenesis toward the VEGF gradient.

volved in the regulation of VEGF and HIF-1α expression through Akt/PKB activity regulation. In addition, the regulation of HIF-1α translational rate has been shown to be through the regulation of mTOR/FRAP, a downstream target of Akt/PKB (Fukuda et al., 2002).

In this paper, we have shown that in human prostate cancer cells, ILK is essential for the regulation of HIF-1α expression and the consequent production of VEGF. Functional inactivation of ILK by exposure to a highly selective chemical inhibitor, or stable or transient transfection of the ILK dominant-negative construct into cell models with high ILK activity, result in a decrease in HIF-1α protein levels and VEGF expression. Furthermore, depletion of ILK protein by siRNA in PC3 cells effectively decreases Akt/PKB and mTOR/FRAP phosphorylation, HIF-1α levels, and VEGF expression. These data suggest that in certain cancer cells, such as prostate carcinoma cells, ILK plays a crucial role in HIF-1α and VEGF expression via activation of PKB/Akt and phosphorylation of mTOR/FRAP (Figure 7). VEGF gene transcription can also be stimulated by the transcription factors AP-1 and NF-κB (Harris, 2002). Since ILK has also been shown to regulate the activities of both of these transcription factors (Troussard et al., 2000; Tan et al., 2002), it is possible that in certain cell types ILK could regulate VEGF expression via signaling pathways that activate these other transcription factors. Although in this study we have not ruled out the contribution of AP-1 and NF-κB in the ILK-mediated regulation of VEGF expression in the prostate cancer cell lines examined, the data presented here support a significant role of HIF-1α in the ILK regulation of VEGF expression. This is particularly true for the PC3 cells in which inhibition of ILK expression or activity results in almost complete suppression of both HIF-1α and VEGF expression. Furthermore, in the PTEN null PC3 cells in which the PI-3 kinase pathway and ILK are constitutively upregulated, transfection of dominant-negative HIF-1α results in substantial inhibition of VEGF expression. It is interesting to note that ILK mRNA has been shown to be upregulated by hypoxia (Scandurro et al., 2001; Grimshaw and Mason, 2001), suggesting that ILK may also play a role in hypoxia-induced VEGF expression. Thus, the role of ILK in hypoxic tumors with constitutive activation of PI-3 kinase may be quite substantial.

We have also shown here that ILK plays an essential role in VEGF-stimulated endothelial cell-mediated blood vessel formation in vitro and in vivo (Figure 6). Migration and proliferation of human endothelial cells in response to VEGF are inhibited upon inhibition of ILK activity or expression. Furthermore, the ability of VEGF-stimulated endothelial cells to form capillary-like structures in vitro is also severely inhibited by inhibiting ILK activity. This inhibition appears to be due primarily to inhibition of cell migration and proliferation, both of which can be regulated by ILK in response to growth factors or engagement of integrins (Wu and Dedhar, 2001; Cruet-Hennequart et al., 2003). The inhibition of angiogenesis in vivo in the CAM assay by inhibition of ILK suggests that the primary effect of ILK inhibition is on endothelial cell migration and ability to form vessels, as there did not appear to be any obvious cell lysis in these assays. This agrees with our finding that HUVEC cell survival appears not to be affected as significantly as cell migration and proliferation upon ILK inhibition. Recent evidence from the systemic and targeted knockout of ILK suggests an important role of ILK in cell adhesion and actin accumulation (Sakai et al., 2003), processes crucial for cell morphogenesis and migration, as well as in cell proliferation (Terpstra et al., 2003).

We have also shown here that inhibition of ILK with a highly selective ILK inhibitor results in the statistically significant suppression of tumor angiogenesis as well as tumor growth in a mouse xenograft model of PC3 tumor growth in SCID mice. These data suggest that inhibitors of ILK activity may be considered as angiogenesis inhibitors effective for the suppression of tumor angiogenesis.

The integrins $\alpha_V\beta_3$, $\alpha_V\beta_5$, and $\alpha_5\beta_1$ have also been shown to be crucial regulators of endothelial cell function during angiogenesis (Hood and Cheresh, 2002; Eliceiri and Cheresh, 2001;

Friedlander et al., 1995). Furthermore, angiogenesis inhibitors such as endostatin and tumstatin have been shown to function by inhibiting integrin function and signaling (Maeshima et al., 2002). Tumstatin has been shown to inhibit endothelial cell survival by binding to $\alpha_V\beta_3$ and inhibiting $\alpha_V\beta_3$-metiated signaling to PKB/Akt (Maeshima et al., 2002). Since ILK is also regulated by integrins and since ILK is involved in $\alpha_V\beta_3$-regualed cell proliferation (Cruet-Hennequart et al., 2003), as well as in regulating anoikis (Attwell et al., 2000), it is likely that ILK also plays an important role in integrin-mediated endothelial cell function during angiogenesis. We have recently created transgenic mice in which the ILK gene is flanked by Lox-P sites and have demonstrated that cells from these mice can be used to conditionally knockout ILK expression in cells isolated from these mice (Troussard et al., 2003). The conditional knockout of ILK in endothelial cells will provide further data on the precise mechanism of ILK function in endothelial cells.

The data shown in this paper demonstrate the importance of ILK in the orchestration of tumor angiogenesis by regulating VEGF expression by carcinoma cells and VEGF-stimulated blood vessel formation. ILK occupies a pivotal position in regulating cell adhesion, actin polymerization, and signaling. A more detailed understanding of these processes is therefore of importance for therapeutic intervention of tumor angiogenesis.

Experimental procedures

Cell culture and transfections

Prostate carcinoma cell lines positive and null for PTEN (DU145 and PC3, respectively) (ATCC) were cultured as suggested by ATCC. PC3 cells were transiently transfected with V5-tagged ILK dominant-negative (ILK-DN:V5), Empty vector, GFP-tagged PTEN (PTEN:GFP), HIF-1α dominant-negative, HIF-1 response element conjugated to GFP reporter (HRE:GFP), and/or Renilla for transfection control using 2–3 μg of cDNA with 6 μl of Lipofectin reagent (Life Technologies Inc.), according to the manufacturer's guidelines. DU145 cells were transfected with 4 μl of Fugene reagent (Roche Molecular Biochemicals). Intestinal epithelial cells (IEC-18) were stably transfected with ILK wild-type (ILK-13 A1a3), ILK dominant-negative (ILK-DN GH31RH), or ILK antisense (ILK-14 antisense) (Hannigan et al., 1996). The parental IEC-18 cells were used as the control. These cells were cultured as previously described (Delcommenne et al., 1998). Human umbilical vein endothelial cells (HUVEC) (ATCC) were cultured as suggested by ATCC. ILK-13 A1a3 and HUVEC cells were transfected with 6 μl of Lipofectamine 2000 reagent (Life Technologies Inc.) according to the manufacturer's guidelines.

Small interfering RNA (siRNA)

PC3 cells were transfected with small interfering RNA (siRNA) that specifically targets the ILK gene (ILK-H or ILK-A) or nonsilencing control using 6 μl of Lipofectin in OPTI-MEM (GibcoBRL) overnight. The cells were passaged 36 hr after incubation in complete media and harvested 36 to 48 hr later as previously described (Troussard et al., 2003). siRNA duplexes were synthesized by Xeragon Inc., MD. Sequences of the human ILK gene specifically targeting the pH domain (ILK-H) (Troussard et al., 2003) and the integrin binding domain (ILK-A) were chosen. The sequence of the DNA target of ILK-A is 5′-GACGCTCAGCAGACATGTGGA-3′. A nonsilencing siRNA (16 base overlap with *Thermotoga maritimia*) was used as the control.

Chemical inhibitors

Cells were exposed to the highly specific small molecule inhibitor (KP-392; Kinetek Pharmaceuticals) (Cruet-Hennequart et al., 2003; Mills et al., 2003; Tan et al., 2001, 2002; Persad et al., 2000, 2001a, 2001b) and equivalent amounts of DMSO vehicle. PI-3 kinase inhibitor LY294002 (Calbiochem) and MEK1 inhibitor PD98059 (Cell Signaling Technology) were dissolved in DMSO as the vehicle.

Western blotting

Cell lyses and Western blotting were carried out as previously described by us (Troussard et al., 2003; Cruet-Hennequart et al., 2003; Tan et al., 2001; Persad et al., 2001a, 2001b). The following antibodies were used in this study: anti-phosphoserine 473 PKB/Akt, anti-PKB/Akt, and anti-phosphoserine 21/9-glycogen synthase kinase-3 (GSK-3) antibodies from New England Biolabs, anti-V5 antibody from Invitrogen, anti-ILK and anti-HIF-1α antibodies from BD Transduction, anti-phosphoserine 2448-mTOR/FRAP and anti-mTOR/FRAP antibodies from Cell Signaling, anti-VEGF antibody from Oncogene Research Products, anti-green fluorescent protein (GFP) antibody from Boehringer Mannheim, and anti-mouse and anti-rabbit antibodies conjugated to horseradish peroxidase from Jackson Laboratories.

Quantification of VEGF in conditioned media

Secreted VEGF was quantified by using an enzyme linked immunoassay (ELISA) kit for human VEGF (Oncogene Research Products) according to the manufacturer's instructions.

ILK kinase assay

HUVECs were starved for 24 hr prior to the experiment. The cells were exposed to increasing amounts of VEGF for 1 or 24 hr, followed by lysis with NP-40 lysis buffer. Equivalent amounts (250 μg) of lysate were immunoprecipitated overnight with 3 mg of mouse monoclonal anti-ILK antibody (Upstate Biotechnologies Institute) at 4°C. The immune complexes were isolated with protein A/G plus agarose beads (Santa Cruz Biotechnology) and washed three times with NP-40 lysis buffer and three times with last wash buffer (50 mM HEPES [pH 7], 2 mM $MgCl_2$, 2 mM $MnCl_2$, 200 mM Na_3VO_4, protease inhibitors). The kinase assay was performed using 2 μg of GSK-3 fusion protein (New England Biolabs) as a substrate, 200 μM ATP in the reaction buffer (50 mM HEPES [pH 7], 2 mM $MgCl_2$, 2 mM $MnCl_2$, 200 mM Na_3VO_4, 200 mM NaF) for 30 min at 30°C. Phosphorylation of the substrate was detected by Western blot with anti-GSK-3 serine 21/9 antibody.

Invasion and migration assay

Invasion and migration was analyzed with a modified Boyden chamber assay (cell culture inserts with a polycarbonate-filter [PVP, 8 μm pore size; Corning Incorporated, New York] covered with growth factor reduced Matrigel [Becton Dickinson, Massachusetts]). Two hundred microliters of cell suspension (5×10^4 cells) were added to the upper wells and allowed to attach for 2 hr at 37°C. Increasing amounts of KP-392 and equivalent amounts of DMSO diluted in 100 μl were added to the upper well for a complete volume of 300 μl. The lower compartment was filled with F-12K medium containing 0 or 20 ng/ml VEGF. Chambers were incubated for 16 hr in a 5% CO_2, 99% humidity, and 37°C atmosphere. Cells on the under side of the filter were quantified by staining the cells with crystal violet, followed by counting the number of cells per magnified field of view (6 fields/membrane).

Cell viability and proliferation assay

The metabolic activity of cells was determined in vitro using the colorimetric cell proliferation/tetrazolium salt WST-1 reagent/Electro Coupling Solution (WST-1/ECS) assay kit (Chemicon International, California) according to manufacturers instructions. The plate was incubated with the reagent for an additional 30 min to 1 hr and quantified by spectrophotometry (OD = 450 nm). All experiments were performed in triplicate.

Immunohistochemical staining

Tissues were frozen in compound-embedding medium (OCT; Miles Inc., Indiana), and 10 μm sections were collected on positively charged slides (Wax-It Histology Services Inc., B.C., Canada). Sections were fixed with cold acetone and blocked with 3% hydrogen peroxidase, followed by 1% bovine serum albumin and normal rabbit serum. Sections were then incubated with the anti-mouse CD31 antibody (BD Pharmingen) overnight at 4°C, followed by incubation with a horseradish peroxidase conjugated anti-rat secondary antibody (Jackson Laboratories) for 1 hr at room temperature. The tissue was washed three times with PBS in between each step. The antibody localization was visualized with NOVARED substrate kit (Vector Laboratories, California), used as directed by manufacturer, and the slides were counterstained with hematoxylin.

Endothelial tube formation assay
The endothelial tube formation assay was performed as previously described with minor modification (Maeshima et al., 2000). A suspension of HUVEC in medium was seeded in triplicate into Matrigel-precoated 24-well plates in the presence of 50 μM KP-392.

Endothelial sprouting assay
Microcarrier beads coated with denatured collagen (Cytodex3; Sigma) were seeded with HUVECs. Fibrin gels were made by dissolving 2.5 μg/μl bovine fibrinogen (Sigma), 0.05 mg/ml Aprotinin (Sigma) in F-12K medium followed by passing the solution through a 0.22 μm pore size filter to sterilize, and mixed with a fraction of HUVEC-coated bead. This mixture was transferred gently to 96-well plates together with HUVEC-coated beads at a density of 25 beads/well with a wide mouth pipette tip. Clotting was induced by adding 1.2 units/ml thrombin. After clotting was complete, F-12K medium containing the indicated inhibitors, equivalent amounts of DMSO vehicle, 0 or 20 ng/ml VEGF, and 1% FBS was carefully applied onto the gel. After 3 days of incubation with daily changes of the medium, the number of capillary-like tubes formed/microcarrier bead (sprouts/bead) was counted by microscopy, monitoring at least 150 beads for each treatment. Only sprouts greater than 150 μm in length and comprised of at least three endothelial cells were counted.

Chorioallantoic membrane (CAM) of chick embryos assay
White Leghorn chicken eggs were fertilized and incubated at 37°C under conditions of constant humidity. The developing CAM was separated from the shell by opening a window at the broad end of the egg above the air sac on embryonic day 6. The opening was sealed with Parafilm (American National Can, Illinois) and the eggs were returned to the incubator. On embryonic day 8, 30 ng/ml VEGF and 50 μM of KP-392 in DMSO or equivalent amounts of DMSO vehicle were loaded onto 2 mm^3 gelatin sponges (Pharmacia Upjohn) and placed on the surface of the developing CAM. Eggs were resealed and returned to the incubator for 10 days as previously described (Roskelley et al., 2001).

PC3 xenograft tumor assay
Six- to eight-week-old male nude mice (Harlan Sprague Dawley, Inc., Indiana) were allowed to acclimatize for 1 week in the Jack Bell Research Centre animal facility. Procedures involving animals and their care conform to institutional guidelines (University of British Columbia Animal Care Committee). One hundred microliters of a PC3 cell suspension (RPMI media [GibcoBRL]; 5% FBS; 2×10^7 cells/ml) was injected subcutaneously into the left and right flank regions of the nude mice via a 27-gauge needle under halothane anesthesia. Ten mice comprised each experimental group. One week post inoculation, animals were dosed daily with the ILK inhibitor (KP-307-2) by intraperitoneal injection of 100 mg/kg of ILK inhibitor (at a concentration of 10 mg/ml in 5% Tween80 in saline). Control mice received equivalent volumes of vehicle (5% Tween 80 in saline). One tumor was removed 1 week post treatment for angiogenesis marker analysis. The Image-Proplus from Media Cybernetics (Carlsbad, California) was used to measure the CD31-positive immunostained endothelial lining (red objects) of neovasculature within the tumors. Tumor growth was monitored three times a week by measuring the height, length, and width of each tumor with a caliper. The tumor volumes were calculated from a formula (axbxc/2) that was derived from the formula for an ellipsoid ($\pi d^3/6$). Data were calculated as the percentage of original (day 1) tumor volume and graphed as fractional tumor volume ± SEM. Mice were sacrificed by day 28 after KP-392 or vehicle administration.

Micrographs
All images were generated using the Nikon Eclipse TE 200 microscope and Nikon Cool PIX 950 digital camera.

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