A Macrophage-Dominant PI3K Isoform Controls Hypoxia-Induced HIF1α and HIF2α Stability and Tumor Growth, Angiogenesis, and Metastasis

Shweta Joshi¹, Alok R. Singh¹, Muamera Zulcic¹, and Donald L. Durden¹,²,³

Abstract

Tumor growth, progression, and response to the hypoxic tumor microenvironment involve the action of hypoxia-inducible transcription factors, HIF1 and HIF2. HIF is a heterodimeric transcription factor containing an inducible HIFα subunit and a constitutively expressed HIFβ subunit. The signaling pathways operational in macrophages regulating hypoxia-induced HIFα stabilization remain the subject of intense investigation. Here, it was discovered that the PTEN/PI3K/AKT signaling axis controls hypoxia-induced HIF1α (HIF1A) and HIF2α (EPAS1) stability in macrophages. Using genetic mouse models and pan-PI3K as well as isoform-specific inhibitors, inhibition of the PI3K/AKT pathway blocked the accumulation of HIFα protein and its primary transcriptional target VEGF in response to hypoxia. Moreover, blocking the PI3K/AKT signaling axis promoted the hypoxic degradation of HIFα via the 26S proteasome. Mechanistically, a macrophage-dominant PI3K isoform (p110γ) directed tumor growth, angiogenesis, metastasis, and the HIFα/VEGF axis. Moreover, a pan-PI3K inhibitor (SF1126) blocked tumor-induced angiogenesis and inhibited VEGF and other proangiogenic factors secreted by macrophages. These data define a novel molecular mechanism by which PTEN/PI3K/AKT regulates the proteasome-dependent stability of HIFα under hypoxic conditions, a signaling pathway in macrophages that controls tumor-induced angiogenesis and metastasis.

Implications: This study indicates that PI3K inhibitors are excellent candidates for the treatment of cancers where macrophages promote tumor progression. Mol Cancer Res; 12(10); 1–12. ©2014 AACR.

Introduction

Hypoxia is considered a hallmark of tumor progression in that it induces neoangiogenesis, the recruitment of bone marrow–derived cells, and degradation of extracellular matrix (ECM) resulting in activation of proliferation, invasion, and metastasis of cancer cells (1). Hypoxic regions of tumors are characterized by an increased accumulation of macrophages which contributes to tumor angiogenesis and tumor progression (2). Hypoxic macrophages are known to respond to hypoxia by upregulating hypoxia-inducible transcription factors (HIF) consisting of distinct, hypoxia-responsive (HIF1α and HIF2α) subunits and identical, constitutively expressed (HIF1β and HIF2β) subunits (3, 4). In the presence of oxygen, the α subunits are hydroxylated by oxygen-sensitive enzymes, prolyl hydroxylases, which targets them for degradation by the von Hippel-Lindau (VHL)-dependent ubiquitin–proteasomal pathway (5, 6). Under hypoxic conditions, HIFα subunits are stabilized, translocate to the nucleus, and, together with their partner factor, basic-helix-loop-helix/PAS protein Arnt, bind to the promoters of genes that mediate glycolysis and neovascularization/angiogenesis (7, 8).

There are conflicting views of the relative contribution of each HIF to the regulation of hypoxic gene expression in macrophages. Some studies suggest that the main form of HIF upregulated by tumor-associated macrophages (TAM) is HIF2 (9, 10), and overexpression of HIF2α in normoxic human macrophages upregulates various proangiogenic genes (11). However, human macrophages also markedly upregulate HIF1α when exposed to hypoxia in vitro and in tumors (12), and HIF1α-deficient murine macrophages express lower levels of such HIF-regulated genes such as VEGF and the glucose receptor GLUT1 in hypoxia than their wild-type (WT) counterparts. Interestingly, the exact contribution of HIF1α and HIF2α to the regulation of hypoxic gene expression appears to vary between different cell types (7).

The molecular mechanisms controlling HIFα accumulation in macrophages are not completely elucidated. In the
tumor cells, a large number of signaling pathways, including Rac-GTP, Src, Fak, PI3K, etc., have been linked to the control of HIFα and VEGF (13, 14). Several groups have implicated the role of PTEN/PI3K signaling in the regulation of HIFα (15–17) in tumor cells. However, the exact contribution of the PI3K pathway to HIFα regulation remains a subject of considerable interest and controversy in both the tumor and stromal compartments (18, 19).

PI3K are a family of enzymes that phosphorylate phosphatidylinositol (PI) lipids at the 3' position (20). The PTEN deleted on chromosome 10 (PTEN) is a dual specificity phosphatase that antagonizes the enzymatic activity of PI3K by dephosphorylating phosphatidylinositol (3,4,5)-trisphosphate to generate phosphatidylinositol (4,5)-biphosphate. PI3K/PTEN has been shown to play a prominent role in regulating a number of cellular responses, including growth, survival, and migration (21, 22). PI3K is known to be associated with different phases of macrophage function (23). The PI3K/Akt signaling axis is crucial for migration and diapause of immune effector cells, such as neutrophils and monocytes, to the site of inflammation and infection (24–26). The role of myeloid-specific PTEN in controlling lung infection and inflammation during murine pneumococcal pneumonia and neutropenia-associated pneumonia is also well-studied (27, 28).

However, the contribution of PTEN/PI3K/AKT pathway in enhancing HIFα-mediated gene expression in macrophages has not been previously reported. In the present study, using myeloid-specific deletion of PTEN and various pan and isoform-specific PI3K inhibitors, we show that PTEN/PI3K signaling controls hypoxia-induced HIFα and HIF2α stabilization in macrophages. Moreover, using p110α−/− mice and pan-PI3K inhibitor SF1126, we demonstrate that PI3K signaling promotes tumor growth and metastasis in tumor models by increasing the expression and potential secretion of proangiogenic factors by TAMs.

**Materials and Methods**

**Animal studies**

All procedures involving animals were approved by the University of California San Diego Animal Care Committee, which serves to ensure that all federal guidelines concerning animal experimentation are met. PI3Kγ (p110γ−/−) mice were obtained from Dr. Joseph Penninger, Institute of Molecular Biotechnology (Vienna, Austria). Floxed PTEN mice and lysozyme M (LysM) Cre recombinase transgenic mice were purchased from Jackson laboratories. LysM Cre mice were mated with PTENfl/fl mice to delete PTEN from myeloid cell compartment. Depending on the presence of LysM Cre recombinase, double-floxed PTENfl/fl mice are referred to as PTENMC-KO or PTENMC-Wt mice, respectively. Macrophages from PTENfl/fl mice were used as WT controls.

**Antibodies and reagents**

Macrophage colony-stimulating factor (MCSF) was from Gibco Life technologies. PI-103, BKM120, and PF4691502 were obtained from SignalRx Pharmaceuticals (29, 30). Primary or fluorescent antibodies against CD31 (clone MEC13.3) and CD11b (clone M17/70) were from BD Biosciences; F4/80 (clone BM8) was from eBiosciences. Alexa Flour 488 was from Invitrogen life Technologies. Bouin’s solution, 4,6-diamidino-2-phenylindole (DAPI), hyaluronidase type V, and DNase I were from Sigma.

**Isolation of bone marrow-derived macrophages, hypoxia and in vitro inhibitor experiments**

Bone marrow-derived macrophages (BMDM) were isolated as described previously and cultured under MCSF (75 ng/mL) + 20% FBS conditions (31). On day 7, most of the adherent cells were macrophages as confirmed by FACS analysis (>90% Mac1- and F4/80-positive cells). To study the effect of different inhibitors in vitro, macrophages (7 days in culture) were treated with or without pan or isoform-specific PI3K inhibitors followed by hypoxia (1% O2) for 4 hours. For hypoxia experiments, macrophages were placed in a modulator incubator chamber (Billups-Rothenberg, Inc.) that was flushed with a gas mixture consisting of 1% O2, 5% CO2, with balance N2, sealed, and incubated at 37°C. Whole-cell lysates were prepared in RIPA buffer (Cell Signaling Technologies) containing complete protease inhibitor cocktail. For HIFα immunobLOTS, nuclear extracts were prepared using Dignam protocol (32).

**Plasmid and siRNA transfections**

BMDMs were transfected using an AMAXA Mouse Macrophage Nucleofection Kit (Lonza) with 5 μg of HA-PTEN or myrAKT plasmid or 100 nmol/L of siRNA for PI3Kα (Mm_pik3ca_1), PI3Kβ (Mm_pik3cb_2), PI3Kγ (Mm_pik3cg_1), PI3Kδ (Mm_pik3cd_1), Akt1/2 siRNA or nonsilencing siRNA (Ctrl_AllStars-1) purchased from Qiagen or Akt1/2 siRNA and control siRNA purchased from Santa Cruz Biotechnology. After transfection, cells were cultured for 36 hours. Each siRNA was tested individually for efficient knockdown of protein expression.

**Quantification of gene expression**

Total RNA was isolated from BMDMs using RNAeasy kit (Qiagen). cDNA was prepared from 1 μg RNA sample using iScript cDNA synthesis kit (Bio-Rad). cDNA (2 μL) was amplified by RT-PCR reactions with 1 × SYBR green supermix (Bio-) in 96-well plates on an CFX96 Real time system (Bio-Rad), using different primers for mouse gene. Sequence of primers used were reported before (31). Relative expression levels were normalized to GAPDH expression according to the formula: 2ΔΔCt, gene of interest – Ct, GAPDH. Values are multiplied by 100 for presentation purposes.

**Immunoblotting**

Whole-cell extract (WCE) or nuclear extracts of BMDMs were prepared and protein was quantitated with Bio-Rad protein assay kit (Bio- Rad Laboratories) using BSA as a standard. Equal protein from protein lysates were resolved by SDS-PAGE, followed by immunoblotting and probing with primary antibodies against p110α (Cell Signaling...
Technologies), p110β (Santa Cruz), p110γ (Cell Signaling Technologies) and p110δ (Santa Cruz), pAKT (Cell Signaling), AKT (Cell Signaling, HIF1α and HIF2α (Novus Biological), and β-actin (Santa Cruz).

In vivo tumor experiments

Lewis lung carcinoma (LLC) and B16 F10 melanoma obtained from the ATCC were cultured as described previously (31). LLC cells (1 × 10⁵) were injected subcutaneously into syngeneic 4- to 6-week-old mice. Tumor dimensions were recorded regularly, and tumors were harvested 25 days postinjection. Tumor volume was measured using the following formula: Volume = 0.5 × length × (width)². For experimental metastasis, B16 F10 melanoma cells (5 × 10⁵) were injected intravenously, and lungs were harvested after 15 days as described before (31). For drug treatment studies, WT mice implanted subcutaneously with 1 × 10⁶ LLC cells were treated with vehicle or 50 mg/kg SF1126 subcutaneously 3 times a week for 28 days, until tumors were harvested. For B16 F10 melanoma, 2 doses of 50 mg/kg of SF1126 were given before injecting 5 × 10⁵ B16F10 melanoma cells, followed by daily treatment with the drug until lungs were harvested on day 15. For survival studies, 3 doses of 50 mg/kg of SF1126 were given before injecting 3 × 10⁵ B16F10 melanoma cells, followed by daily treatment until mice died. Tumor volumes and weights were measured and the tumors were used for immunofluorescence, IHC, and sorting of macrophages using FACS. For FACS analysis, LLC tumors were excised, minced, and digested to single-cell population as described (31). CD11b- and F4/80-positive cells sorted by FACS were used for RNA isolation and real-time PCR studies.

Results

PTEN regulates expression of HIFα protein and HIFα mRNA targets in macrophages

The tumor suppressor PTEN is an antagonist of PI3K signaling and functions by removing the phosphate at the D3 position of phosphatidylinositol trisphosphate and phosphatidylinositol bisphosphate. To validate the role of PTEN in hypoxia-induced HIFα stabilization, we generated conditional PTEN knockout mice in which PTEN expression was controlled by LysM, a myeloid cell–specific promoter (20). Depending on the presence of LysM Cre recombinase, double-floxed PTEN+/β/ mice are referred to as PTENMC-KO or PTENMC-WT mice, respectively. Deletion of PTEN was confirmed in primary macrophages (Fig. 1A), and resulting downstream effects of constitutively active PI3K

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**Figure 1.** Deletion of PTEN in myeloid cells increase hypoxic HIF1α and HIF2α accumulation. A, Western blot analysis showing deletion of PTEN in myeloid cells. Cell lysates from BMDMs isolated from PTENMC-WT or PTENMC-ko mice were subjected to Western blot analysis for PTEN, pAKT, and AKT. β-Actin was used as loading control. B and C, BMDMs isolated from PTENMC-WT and PTENMC-ko (B) and PTEN+/+ and PTEN–/– (C) mice were incubated under hypoxic (1% O₂) conditions for 4 hours followed by preparation of nuclear extracts (for HIF1α and HIF2α) and Western blot analysis. Each lane in A, B, and C represents lysate prepared from individual mice. Mouse ID numbers are provided for each genotype. D, mRNA was isolated from PTENMC-WT and PTENMC-ko BMDMs incubated under hypoxia (1% O₂) for 24 hours. VEGF mRNA expression was measured by real-time PCR. Data represent mean ± SEM (n = 3 or 4; P < 0.001; pairwise 2-sided Student t test). E, BMDMs isolated from PTENMC-ko mice were transfected with 5 μg of HA-PTEN, using AMAXA mouse macrophage Nucleofaction kit. After 36 hours of transfection, cells were exposed to normoxia (21% O₂) or hypoxia (1% O₂) for 4 hours followed by preparation of either nuclear extracts (for HIFα isoforms) or WCE (PTEN blot) and Western blot analysis. Experiments were repeated 4 to 5 times with 2 to 3 mice in each group.
were reflected by greatly elevated baseline levels of phospho-Akt in PTENMC-KO macrophages (Fig. 1A). Myeloid-specific deletion of PTEN induced high levels of HIF1α and HIF2α stabilization in PTENMC-KO mice as compared with PTENMC-Wt (Fig. 1B). These results were also supported by a greater degree of HIFα stabilization in BMDMs isolated from PTENMC-KO mice (Fig. 1C). Our next point of focus was to investigate the role of PTEN in the transcriptional activation of the HIFα target gene VEGF. Loss of PTEN in myeloid cell compartment increased VEGF mRNA levels in PTENMC-KO mice (Fig. 1D). To further validate the role of PTEN in stabilization of HIFα, PTEN was exogenously expressed in PTENMC-ko mice. Results in Fig. 1E showed that expression of PTEN blocked the accumulation of HIFα in PTENMC-ko mice. Taken together, these results suggest a role for PTEN in the regulation of the HIFα/VEGF axis under hypoxia in macrophages (Fig. 1).

Inhibition of PI3K/AKT pathway blocks the hypoxic induction of HIF1α and HIF2α and its transcriptional target VEGF in macrophages

The observation that PTEN exerts control over HIF1α and HIF2α protein expression and transcriptional activity (Fig. 1) led us to investigate the effects of different clinically relevant PI3K inhibitors (SF1126, PF4691502, PI-103, and BKM120) on the hypoxic induction of HIFα in macrophages. SF1126, a pan-PI3K inhibitor, is a vascular RGDS/integrin targeted prodrug derivative of LY294002 and is entering phase II clinical trials (29). The other inhibitors used in early-phase clinical trials include: PI-103, a dual PI3K/mTOR inhibitor; PF4691502, a pan-PI3K inhibitor; and BKM120, a pan-PI3K inhibitor (33–35). The results shown in Fig. 2A clearly demonstrate that SF1126, PI-103, PF4691502, and BKM120 potently inhibit the hypoxic induction of HIF1α and HIF2α in BMDMs. Furthermore,
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these pan-Pi3K inhibitors also blocked the transcriptional activation of HIFα target gene, VEGF (Fig. 2B). In addition to pan-Pi3K inhibitors in clinical trial, we used SF2523, a novel pan-Pi3K inhibitor developed in collaboration with SignalRx Pharmaceuticals (30), to see the effect of this Pi3K inhibitor on HIFα stabilization. SF2523 blocked accumulation of HIF1α and HIF2α in dose-dependent manner (Fig. 2C), which correlates with the decreased VEGF mRNA in BMDMs treated with SF2523 (Fig. 2D). To determine the role of Akt in hypoxic HIFα accumulation, experiments were performed where Akt is either knocked down or an activated membrane-targeted Akt (myrAkt) is exogenously expressed in BMDMs. Figure 2E and F showed that knockdown of Akt using siRNA blocked accumulation of HIFα, whereas exogenous expression of myrAkt increased HIFα levels.

Specific p110δ and p110γ isoforms of Pi3K control HIFα/VEGF axis in macrophages

We next focused on the specific isoforms of Pi3K involved in regulating the HIFα/VEGF axis in macrophages. Recently, published reports (26) and our results provide evidence that p110γ is the predominant isoform expressed in the myeloid cells and macrophages (Supplementary Fig. S1A). To determine the specificity of the Pi3K signaling in HIFα accumulation isoform-specific Pi3K inhibitors, namely, GDC-0941 (p110γ-specific inhibitor; ref. 36), TGX-221 (p110β-specific inhibitor; ref. 37), Cal101 (p110δ-specific inhibitor; ref. 38) and AS605240 (p110γ-specific inhibitor; ref. 39) were used. Interestingly, we found that AS605240 (a p110γ isoform inhibitor) showed the most marked inhibition of HIFα accumulation and decreased the transcriptional activation of VEGF in BMDMs, whereas the effect of inhibitors of α, β, and δ isoform did not show a marked effect on HIFα accumulation (Fig. 3A and B). However, inhibitors of α, β, and δ isoform significantly blocked VEGF mRNA expression. Although isoform-specific inhibitors of Pi3K display some level of selectivity, the kinase inhibitory profiles and IC50 potencies against different p110 isoforms are not absolute, making conclusions difficult. To confirm a specific role for different Pi3K catalytic isoforms in the regulation of HIFα accumulation, we therefore knocked down the expression of p110 isoforms in BMDMs by using siRNA against these isoforms. As shown in Fig. 3C and D, targeting of specific p110γ isoform led to decreased hypoxia-induced HIFα stabilization and reduced expression of VEGF mRNA, whereas there is no effect on HIFα protein and VEGF mRNA levels on targeting α and β isoforms. Interestingly, knockdown of p110δ isoform also blocked accumulation of HIFα protein as well as VEGF gene expression (Fig. 3C and D). Taken together, these results support that HIFα stability is mainly contributed by p110γ isoform and partially by p110δ isoform of Pi3K. To validate our results that p110γ is the major isoform involved in regulating HIFα stability, we used p110γ-/− mice. The BMDMs isolated from these mice show decreased HIFα accumulation and VEGF secretion (Fig. 3E and F). However, p110γ−/− BMDMs treated with AS605240 (p110γ-specific inhibitor) did not show any further reduction in VEGF levels (Fig. 3F), further validating our results that p110γ is the major isoform involved in HIFα accumulation and VEGF secretion.

Blocking Pi3K signaling axis induces the hypoxic degradation of HIFα via the 26S proteasome pathway

The above results (Figs. 1–3) clearly establish a key role for the PTEN/Pi3K signaling pathway in hypoxia-induced HIFα accumulation in macrophages. To explore the mechanism by which PTEN/Pi3K pathway affects HIFα levels in macrophages, we used the proteasome inhibitor, MG132 to ask whether this process was related to proteasome-dependent degradation of HIF. BMDMs from PTENMC-WT and PTENMC-KO mice were treated with the proteasome inhibitor, MG132, before exposure to hypoxia. Our data support the hypothesis that PTEN affects HIFα under hypoxic conditions by inducing the hypoxic degradation of HIFα as this effect can be reversed by pretreatment with the proteasome inhibitor, MG132 (Fig. 4A). To further confirm that the disappearance of HIFα is a proteasome-dependent event and that Pi3K activation is required for the hypoxic stability of HIFα, we treated macrophages with a panel of Pi3K inhibitors. The decrease in HIFα accumulation observed in BMDMs treated with pan-Pi3K inhibitors, p110γ isoform-specific Pi3K inhibitor, AS605240, and novel pan-Pi3K inhibitor, SF2523, was reversed by treatment with MG132 (Fig. 4B–D). The degradation of HIFα by VHL under normoxic conditions is well known; however, its degradation under hypoxia is poorly understood. Importantly, our results provide direct evidence that HIFα is degraded under hypoxic conditions in a PTEN- and Pi3K-dependent manner in macrophages.

Pi3Kγ signaling in macrophages promotes tumor growth and metastasis

It is well documented that macrophages exposed to hypoxia accumulate both HIF1α and HIF2α, and overexpression of HIF2α in TAMs is specifically correlated with high-grade human tumors and poor prognosis (9). On the same note, Doedens and colleagues reported that macrophage expression of HIF1α suppresses T-cell function and promotes tumor growth (40). On the basis of the results observed in the present study showing the important role of Pi3K signaling axis in controlling both HIF1α and HIF2α accumulation in macrophages, we hypothesize that Pi3K signaling should promote tumor growth by increasing secretion of proangiogenic factors in TAMs. To validate this hypothesis, syngeneic LLC was injected subcutaneously into WT and p110γ−/− mice. Tumor growth and angiogenesis were drastically reduced in p110γ−/− mice (Fig. 5A and B). Immunofluorescence CD31 staining showed that the microvessel density was significantly reduced in tumors propagated in p110γ−/− mice (Fig. 5B). On the basis of previously published report that p110γ is the isoform predominantly expressed in myeloid cell compartment (26) and our observation that p110γ isoform is the major contributor of

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hypoxia-induced both HIF1α and HIF2α accumulation in macrophages (Fig. 3C–F), we hypothesized that macrophages infiltrating into the tumors implanted in p110γ−/− mice would be defective in secretion of proangiogenic factors that promote tumor growth. To validate this hypothesis, macrophages were FACs sorted (on the basis of CD11b and F4/80 staining) from LLC tumors implanted in WT and p110γ−/− mice and evaluated for expression of genes required for tumor growth and progression. For this, a panel of TAM-associated genes, VEGF, matrix metalloproteinase (MMP)9, urokinase-type plasminogen activator (uPA), COX2, arginase, MMR, TGFβ, IL1, and TNFα, was used to examine the gene expression profile of macrophages present in the tumor microenvironment of WT and p110γ−/− mice. The expression levels of COX2, uPA, MMP9, MMR, arginase, and VEGF are significantly higher in macrophages sorted from LLC tumors of WT than in p110γ−/− mice (Fig. 5C). In contrast, the macrophages sorted from tumors implanted in p110γ−/− animals showed higher levels of IL1 and TNFα (Fig. 5C). Consistent with this, existing literature suggests that TAMs secrete several proteins including MMP-9 (41), uPA (42), and VEGF (43), which promote tumor growth, angiogenesis, and metastasis. IL1 and TNFα are proinflammatory cytokines which are reported to be expressed at a lower level in tumor-associated M2 macrophages and are known to promote tumor suppression (44). These results suggest that macrophages isolated from tumors implanted in p110γ−/− mice express higher levels of proinflammatory cytokines seen in M1 macrophages which promote tumor suppression, whereas macrophages isolated from WT mice show higher expression of genes which promote tumor growth and progression. Tumor growth and angiogenesis are prerequisites for metastasis; hence, we examined whether p110γ−/− mice display
any defect in metastasis. To explore the role of p110γ in experimental lung metastasis, B16F10 melanoma cells were injected into mouse tail veins of WT and p110γ−/− animals. Two weeks later, we observed massive numbers of metastatic foci in the lungs of WT mice compared with a marked reduction in number of nodules in p110γ−/− mice (Fig. 5D). A microscopic analysis of lung tissue sections confirmed the results from gross specimens and demonstrated larger numbers of metastatic foci in the lungs of WT mice (Fig. 5D). These observations led us to examine whether the BMDMs prepared from p110γ−/− mice in vitro display any defect in expression of genes promoting tumor growth and progression. We found that macrophages isolated from LLC tumors growing in p110γ−/− mice were defective in the expression of genes previously implicated in the promotion of tumor growth (M2 markers; Fig. 5E) including VEGF described in Fig. 3F.

**SF1126, a pan-PI3K inhibitor, blocks tumor growth and metastasis and reduces the expression of proangiogenic factors by TAMs**

Considering the role of PTEN and PI3K signaling in the macrophage-dependent promotion of tumor growth and tumor-induced angiogenesis, we examined the effects of a pan-PI3K inhibitor, SF1126, in the LLC model for its effects on tumor growth, angiogenesis, and metastasis in vivo (Fig. 6). SF1126 has completed phase I clinical trial in solid tumors and B-cell malignancies and is headed for a phase II trial in 2014 (29). The administration of 50 mg/kg dose of SF1126, 3 times a week, significantly blocked tumor growth in WT animals implanted with LLC (Fig. 6A). Hence, we determined whether SF1126 exerts an effect on the neovascularization of these tumors. Immunofluorescence CD31 staining showed that the microvessel density was significantly reduced in the mice treated with SF1126 (Fig. 6B). Because TAMs are known to have a marked influence on the regulation of tumor-induced angiogenesis (45), we propose that SF1126 blocks angiogenesis in the tumors by blocking the proangiogenic factors secreted by TAMs within the tumor microenvironment. The supporting evidence for this notion comes from the real-time PCR data obtained from macrophages (FACS sorted on basis of CD11b and F4/80 staining) isolated from the LLC tumors implanted into WT mice and treated with or without SF1126 (Fig. 6C). Interestingly, the macrophages sorted from the mice treated with SF1126 inhibitor showed reduced expression of VEGF, MMP9, arginase, and COX2 (Fig. 6C). The results in Fig. 6C suggest that macrophages isolated from SF1126-treated tumors display a reduced expression of proangiogenic factors promoting metastasis. So, we next explored whether the administration of SF1126 can block tumor metastasis. We observed a marked increase in metastatic foci in the lungs of WT mice compared with the 60% reduction in metastatic nodules seen in SF1126-treated mice injected with B16F10 melanoma (P < 0.01; Fig. 6D and E). Our results demonstrating that SF1126 blocked metastasis lead us to explore the effect of SF1126 on
survival in the B16 experimental metastasis model. The data in Fig. 6F demonstrate that SF1126 treatment prolongs the survival of mice by average 15 days, a result which directly correlates with the inhibition of experimental metastasis in this model and validating the efficacy of this drug either alone or in combination with other drugs in the treatment of cancer. Taken together, we conclude that SF1126 blocks tumor growth and metastasis potentially by blocking HIF1α and HIF2α and the downstream expression and secretion of proangiogenic factors by tumor-infiltrating M2 macrophages. On the basis of these results, we propose a hypothetical model in which E3 ligase degrades HIF1α under hypoxic conditions in the cytoplasm in a proteasome-dependent manner. The model would predict that the stimulation of PI3K and AKT via upstream signals will serve to promote translocation of E3 ligase into the nucleus and hence promote the stabilization of hypoxic HIF1α. The pathway will bring hypoxic HIF1α under the control of the PTEN/PI3K/AKT signaling axis to regulate an E3 ligase to control hypoxic HIF1α levels and angiogenesis and tumor metastasis in vivo (Fig. 7).

Discussion

It is well established that TAMs accumulate in hypoxic regions of tumors and that hypoxia triggers a proangiogenic program in these cells stimulating the production of angiogenic factors and matrix-regulatory proteins such as VEGF and MMPs, respectively (46, 47). Therefore, macrophages
recruited in situ represent an indirect pathway of amplification of angiogenesis, in concert with angiogenic molecules directly produced by tumor cells. Thus, we speculate that targeting the common pathway promoting angiogenesis in macrophages as well as tumor cell compartment will likely provide more effective therapy in the treatment of cancer.

The PI3K pathway is known to play a critical role in tumor cell survival, angiogenesis, invasion, apoptosis, and cellular metabolism (15, 22) and is considered as the most frequently activated pathway in cancer. Herein, we provide direct evidence that PI3K pathway controls tumor growth and angiogenesis and regulates the HIFα/VEGF axis in macrophages. Moreover, SF1126, a pan-PI3K inhibitor entering phase II clinical trials, blocks macrophage-mediated tumor growth, angiogenesis, and metastasis, suggesting efficacy of SF1126 and other PI3K inhibitors in the treatment of cancer in which macrophages are important in tumor progression.

Recent studies have clearly demonstrated that HIF1α and HIF2α are stabilized in hypoxic macrophages in vitro (12). Talks and colleagues demonstrated that HIF2α protein accumulates at high levels in TAMs detected in various human cancers (9). Recent reports suggest the important role...
of HIF2α in macrophage functions in mouse models of tumor inflammation (48). In the present study, using genetic mouse models, siRNA approach, and clinically relevant PI3K inhibitors, we provide evidence that PTEN/PI3K signaling controls both HIF1α and HIF2α accumulation in macrophages. The role of PTEN/PI3K signaling in promoting HIFα stabilization in tumor cells is well-documented. However, there are no reports examining the role of this important signaling axis in HIFα stabilization in macrophages. Our studies clearly demonstrate that the loss of PTEN in the myeloid cell compartment promotes hypoxic HIFα stabilization in BMDMs (Fig. 1). Our studies provide convincing evidence that the expression of PTEN and inhibition of PI3K/AKT signaling induces the hypoxic degradation of HIF1α and HIF2α in a proteasome-dependent manner. While much is known about the transcriptional and posttranslational regulation of HIFα, there is very limited evidence that HIFα can be degraded under conditions of hypoxia by the 26S proteasome (49). Liu and colleagues recently described oxygen-independent degradation of HIF1α by a novel HIF1α-interacting protein, receptor for activated C-kinase 1 (RACK1; ref. 50). They suggested that similar to the E3 ligase pVHL, RACK1 increased polyubiquitination of HIF1α and was unable to mediate degradation of HIF1α in the presence of proteasomal inhibitor MG132. In the same context, recent study suggests that SHARP promotes proteasomal degradation of HIF1α independent of pVHL, hypoxia, and ubiquitination machinery (51). Recently published work from our laboratory performed in glioma tumor models has demonstrated that PTEN and PI3K inhibitors control the hypoxic degradation of HIF1α and that the E3 ligase, MDM2, is required for hypoxic degradation of HIF1α (52). The E3 ligase and other signaling pathways controlling the degradation of HIF1α and HIF2α under hypoxic conditions in macropages remain an active area of investigation and will be a major focus of our future research efforts.

Moreover, we observed that p110γ, the most dominant isoform of PI3K expressed in macrophages, controls HIF1α and HIF2α accumulation in macrophages. Interestingly, our data provide evidence that p110δ isoform contributes significantly to HIFα stabilization and VEGF secretion in BMDMs (Fig. 3C and D). More importantly, we demonstrate that the expression of p110γ in macrophages promotes tumor growth and metastasis potentially by controlling the expression of proangiogenic and tumor growth-promoting factors by TAMs (Fig. 5C and E). A recent report by Schmid and colleagues has shown that p110γ promotes tumor inflammation by controlling the trafficking of myeloid cells into tumor (26). Our work extends these findings and...
suggestions that the expression of p110γ in macrophages controls the hypoxia-driven HIFα/VEGF axis which plays a crucial role in neoangiogenesis and tumor growth. Moreover, our studies provide evidence that administration of SF1126 blocks marker degradation in the tumor stromal macrophage compartment as it blocks tumor growth, angiogenesis, and metastasis in vivo. In conclusion, the major findings of this article are: (i) we identified an important role of PTEN/PI3K/AKT signaling axis in HIFα and HIFα stabilization in macrophages; (ii) PTEN and several clinically relevant PI3K inhibitors exert their control over HIFα/VEGF axis by inducing the hypoxic degradation of HIFα subunit in a proteasome-dependent manner; (iii) expression of p110γ in macrophages promotes tumor growth and metastasis; and (iv) SF1126 potently blocked macrophage-mediated tumor angiogenesis and metastasis. These studies suggest that SF1126 and other PI3K inhibitors are excellent candidates for the treatment of cancers where macrophages promote tumor progression.

Disclosure of Potential Conflicts of Interest
D.L. Durden discloses a financial conflict of interest relating to the development of SF1126. D.L. Durden has ownership interest (including patents) in SignalRx Pharmaceuticals. This relationship has been reviewed by the UCSD committee on conflict of interest. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Zulcic
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