Oncogene (2010) 29, 2550–2559

www.nature.com/onc

ORIGINAL ARTICLE

PI4KIIα is a novel regulator of tumor growth by its action on angiogenesis and HIF-1α regulation

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Tumor growth is the orchestration of various oncogenes and tumor suppressors, and the regulation of these genes offers a rational therapeutic approach to cancer treatment. In this study, we found a new regulator of tumor growth, phosphatidylinositol 4-kinase type IIα (PI4KIIα), the mechanism of which is involved in angiogenesis and hypoxia-inducible factor HIF-1α regulation. Results obtained from a human cancer tissue microarray showed that PI4KIIα protein expression increases markedly in seven types of cancers compared with normal tissues. Suppression of PI4KIIα leads to retarded tumor growth in nude mice. Downregulation of PI4KIIα in cancer cells eliminates tumor cell-induced endothelial cell tubulogenesis and migration, and results in impaired angiogenesis. Further investigation showed that PI4KIIα can directly regulate HIF-1α expression and that the expression of these two proteins is correlated *in vivo*. At the same time, downregulation of PI4KIIα markedly reduces HER-2 autophosphorylation, and PI4KIIα specifically triggers HIF-1α accumulation through a phosphatidylinositol 3-kinase (PI3K)- and extracellular signal-regulated protein kinase (ERK)-dependent pathway, suggesting that PI4KIIα may regulate HIF-1α through the HER-2/PI3K, ERK cascade. In summary, we discovered a pivotal role for PI4KIIα in the regulation of tumor growth. Our results shed new light on understanding the novel functions of PI4KIIα in cancer and suggest that PI4KIIα may be a promising specific target for tumor therapy.
Oncogene (2010) **29,** 2550–2559; doi:10.1038/onc.2010.14; published online 15 February 2010

Keywords: phosphatidylinositol 4-kinase type IIα (PI4KIIα); tumor; angiogenesis; hypoxia-inducible factor-1α (HIF-1α)

Introduction

Phosphatidylinositol 4-kinases (PI4Ks) are best known for providing the substrate for phosphatidylinositol 3-kinase (PI3K) and for producing second messengers (Gehrmann and Heilmeyer, 1998; Heilmeyer *et al*., 2003; Di and De, 2006). Two kinds of PI4Ks have been characterized, namely type III phosphatidylinositol (PI) 4-kinases (PI4KIIIα and PI4KIIIβ) and type II PI 4-kinases (PI4KIIα and PI4KIIβ) (Heilmeyer *et al*., 2003). Recent evidence suggests that PI(4)P produced by PI4KIIα has an independent function in its own right beyond that of generally providing a substrate for PI3K. It has been reported that PI4KIIα is important for Wnt signaling in *Xenopus* embryos and interacts with Dvl (Pan *et al*., 2008; Qin *et al*., 2009). Waugh *et al*. (2003) reported that PI4KIIα colocalized with valosin-containing protein, a potential prognostic marker for cancer. Xu *et al*. (2006) found that PI4KIIα is associated with cellugyrin-positive Glut4 vesicles. PI4KIIα has also been shown to be essential in both adaptor complex-1- and adaptor complex-3-dependent membrane trafficking (Wang *et al*., 2003; Craige *et al*., 2008). A recent report showed that PI4KIIα associates with Hermansky–Pudlak syndrome protein complexes (Salazar *et al*., 2009). PI4KIIα is necessary for endosomal trafficking and degradation of activated epidermal growth factor receptor (EGFR) (Minogue *et al*., 2006), and it is also a critical player in the exo-endocytic cycle of synaptic vesicles (Guo *et al*., 2003). The loss of PI4KIIα in mouse causes late onset degeneration of spinal cord axons (Simons *et al*., 2009). On the basis of these reports, it can be seen that research on PI4K functions in its own right is attracting a significant amount of attention.

Of the PI4KIIα functions described above, those involving the Wnt signaling pathway, valosin-containing protein and EGFR all have important roles in tumor progression (Hsuan, 1993; Koesters and von Knebel, 2003; Yamamoto *et al*., 2004). In addition, a previous study has shown that most PI kinases have higher activities in cancer cells than in normal cells. Total PI4K activity is also higher in cancer cells (Weber *et al*., 1996). Taken together, these results imply that PI4KIIα could be involved in tumor progression. However, the precise function of PI4KIIα in cancer is not known at present.

Multiple genetic mutants contribute to tumor growth either by regulating the proliferation of cancer cells, or by changing the microenvironment of tumor, such as angiogenesis (Hanahan and Folkman, 1996). Inhibition

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Received 5 August 2009; revised 25 December 2009; accepted 6 January 2010; published online 15 February 2010

of tumor angiogenesis has been regarded as an attractive nontoxic means of regulating tumor progression (Folkman, 1971). One of the key mediators of tumor-induced angiogenesis is hypoxia-inducible factor-1 (HIF-1), a transcription factor that controls the expression of target genes correlated with tumor angiogenesis, such as *vascular endothelial growth factor (VEGF)* and *inducible nitric oxide synthase (iNOS)* (Melillo *et al*., 1995; Ferrara, 2002; Lee *et al*., 2004). The activity of HIF-1 is mainly regulated by the state of its O_2-regulated α-subunit (HIF-1α), and the steady-state level of HIF-1α protein is determined by its level of synthesis and its stability that are regulated by the PI3K and mitogen-activated protein kinase pathways and proteasomal degradation, respectively. HIF-1α has an important role in the regulation of tumor angiogenesis (Lee *et al*., 2004).

In this investigation, our aim was to explore the roles of PI4KIIα in tumor growth. Our data show that specific knockdown of PI4KIIα inhibited tumor growth, and this inhibition is correlated with HIF-1α accumulation. Our findings suggest that PI4KIIα may be a promising specific therapeutic target for tumor control.

Results

PI4KIIα is correlated with and has a crucial role in tumor growth

To study the function of PI4KIIα in cancer, we first investigated PI4KIIα and HIF-1α expression at different time points during tumor growth using a xenograft tumor assay. MCA205 fibrosarcoma cells, HeLa cells and MCF-7 cells were inoculated subcutaneously in BALB/c nude mice, and tumor growth was monitored (Figure 1a). Parallel to the progression of tumor growth, the expression of PI4KIIα was markedly induced in the three types of tumors (Figure 1b). At the same time, we also measured the PI4KIIβ expression along with tumor growth and found that the PI4KIIβ expression increased in MCA205 and MCF-7 cell xenografted tumors, but did not change in HeLa cell xenografted tumors (Supplementary Figure S1). The expression of HIF-1α in tumor tissues was examined at the same time as a positive control. Consistent with previous reports (Semenza, 2001, 2002), HIF-1α increased significantly during tumor growth (Figure 1b). These results show that both PI4KIIα and HIF-1α are simultaneously upregulated with tumor growth.

To confirm the relationship between tumor growth and PI4KIIα, we examined PI4KIIα expression levels in a 500-core tissue microarray with >18 of the most common types of cancer (nearly 20 cases per type) and normal controls (~ 5 cases per type) from 495 individual patients. Immunohistochemical staining was performed on a high-density multiple organ cancer and normal tissue array with anti-PI4KIIα. Results for bladder transitional cell carcinoma and nonmalignant normal bladder tissue from the tissue microarray were used as standards for comparison (Supplementary Figure S2A). All tissues were scored according to this scheme, and the data are summarized in Table 1. The results indicate that almost all cancer tissues have high PI4KIIα expression and that PI4KIIα expression is significantly upregulated compared with the corresponding normal tissue in human breast nonspecific infiltrating duct carcinoma, breast infiltrating lobular carcinoma, thyroid papillary carcinoma, thyroid follicular carcinoma, fibrosarcoma, malignant melanoma (dermis) and bladder transitional cell carcinoma. A representative example is shown in Figure 1c. Consistent with a previous report (Minogue *et al*., 2001), the tissue microarray showed that PI4KIIα is ubiquitously expressed in human tissues, with levels being higher in the brain, kidney, stomach and lung tissues, and lower in thyroid, fibrous tissue, dermis and breast tissues. Taken together, on the basis of the above observation, we hypothesize that PI4KIIα is correlated with tumor growth and may have an important role in tumor progression.

To identify the site of upregulated PI4KIIα in tumor tissues, we conducted PI4KIIα and hematoxylin–eosin staining on a series of normal and cancerous tissue sample slices collected from patients with breast cancer. As shown in Figure 1d, upregulated PI4KIIα was strictly located in the cytosol of cancer cells, but was not present in other cell types. The staining of a HeLa cell-induced tumor showed the same result (Supplementary Figure S2B).

To determine whether the upregulated expression of PI4KIIα really has a role in tumor growth, a PI4KIIα downregulated HeLa cell line was generated using an RNA interference technique. PI4KIIα small interfering RNA (siRNA) HeLa cells were injected subcutaneously into one side of the venter of nude mice. As a control, the same amount of control RNA interference-transfected HeLa cells was injected into the opposite side of the same mice. As shown in Figure 2a, the growth of PI4KIIα siRNA HeLa tumors was strongly suppressed compared with that of control siRNA HeLa tumors; on day 18 after tumor cell inoculation when the mean volume of control siRNA HeLa tumors was already more than $1100\,mm^3$, the volume of PI4KIIα siRNA HeLa tumors was only $\sim 500\,mm^3$. To exclude the possibility that this effect was restricted to HeLa cells, PI4KIIα expression was also suppressed in MCF-7 human breast carcinoma cells and HEK-293T cells; downregulation of PI4KIIα expression in both types of cells also led to a marked retardation of tumor growth in nude mice (Figure 2b and c). We also investigated the expression of PI4KIIα and HIF-1α in both PI4KIIα knockdown tumors and control tumors on the fifteenth day of transplantation. As shown in Figure 2d, both PI4KIIα and HIF-1α were inhibited in PI4KIIα knockdown tumors, indicating that the siRNA for PI4KIIα can last more than 15 days. These results clearly show that blocking PI4KIIα expression in tumor cells suppresses tumor growth *in vivo*. Thus, we can conclude that PI4KIIα has an important role in tumor progression.

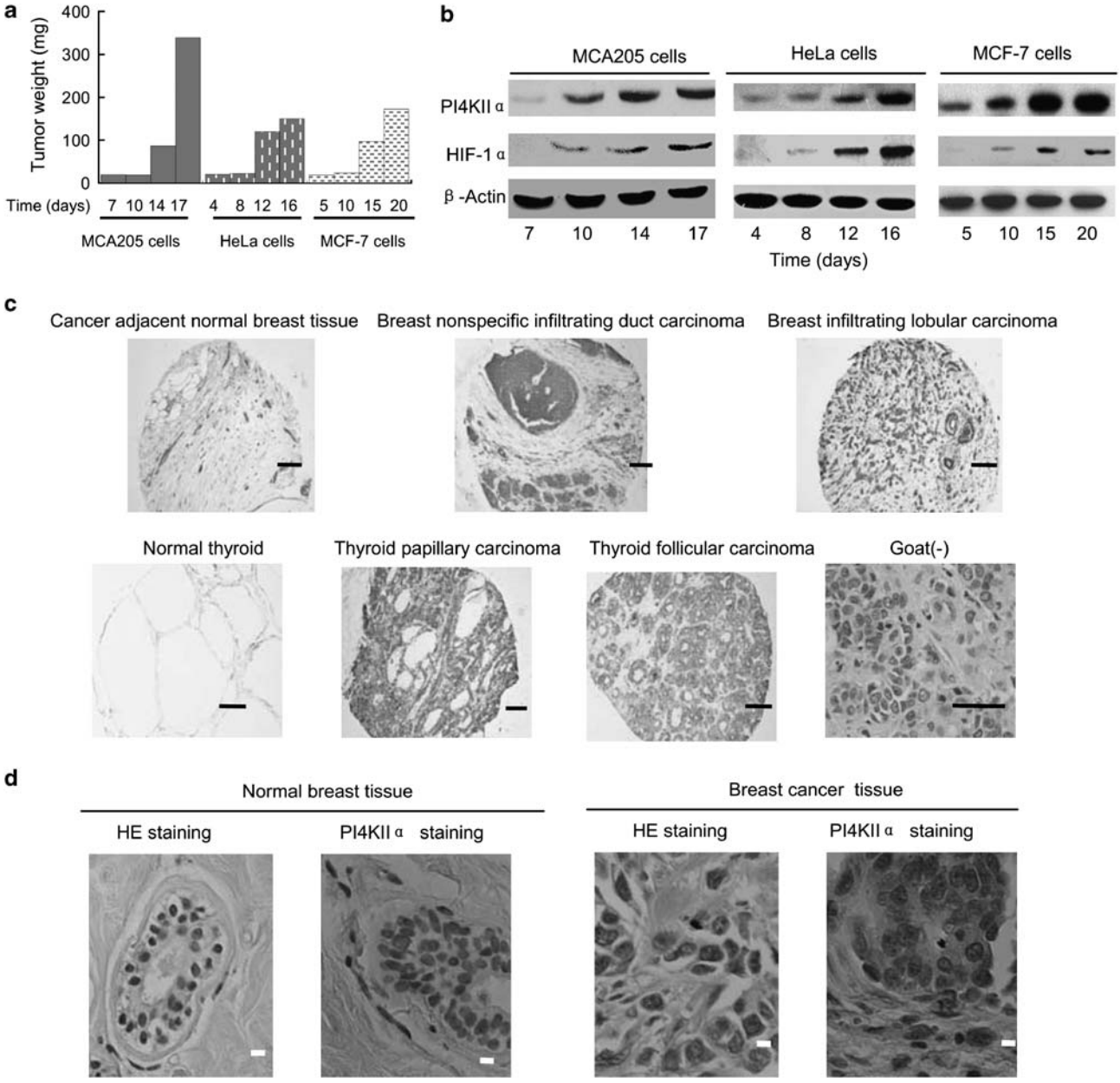

Figure 1 PI4KIIα is correlated with tumor growth. BALB/c nude mice (6 mice per group) were subcutaneously injected with 5×10^6 HeLa cells or MCF-7 cells or MCA205 cells in the venter. After the length of time indicated, tumors were isolated, lysed with RIPA buffer and examined by western blotting. (**a**) Tumor weight changes with time. (**b**) Tumor tissue expression of PI4KIIα and HIF-1α over time. All figures represent two independent experiments. (**c**) Representative examples of PI4KIIα expression in indicated tumor types compared with the corresponding normal tissue in the tissue microarray using immunohistochemical staining with anti-PI4KIIα. Goat controls demonstrated no staining. (**d**) HE and PI4KIIα staining in a serial section of patient breast cancer tissue and normal tissue, respectively. Black bar means 100 μm; white bar means 10 μm.

PI4KIIα regulates tumor-induced angiogenesis through endothelial cell migration and tube formation

To further investigate the mechanism by which downregulation of PI4KIIα inhibits tumor growth, we first compared the viability of cells overexpressing PI4KIIα (HeLa) and cells downregulated for PI4KIIα expression (HeLa, MCF-7 and HEK-293 cells) with the control using an MTT assay. Results showed that regulation of PI4KIIα expression had no obvious impact on cell growth within 48 h. In addition, we tested the toxic effects of PI4KIIα siRNA on HeLa cells and MCF-7 cells using the LDH assay. Consistent results indicating that the downregulation of PI4KIIα expression did not show significant toxicity on both cells within 48 h were obtained (Supplementary Figure S3). We then investigated another important factor for tumor growth, namely angiogenesis, by quantifying the hemoglobin content in growing tumors. As shown in Figure 3a, the concentration of hemoglobin in PI4KIIα siRNA HeLa tumors was much less than that in control tumors.

Table 1 The PI4KIIα protein level in relevant normal and cancer tissues

Normal tissue			*Cancer tissue*		
Tissue type	*PI4KIIα level low/total*[a]	*PI4KIIα level high/total*[a]	*Pathology diagnosis*	*PI4KIIα level low/total*[a]	*PI4KIIα level high/total*[a]
Skin (dermis)	10/10	0/10	Malignant melanoma (dermis)	1/20	19/20
Cancer adjacent normal breast tissue	9/9	0/9	Breast nonspecific infiltrating duct carcinoma	2/18	16/18
			Breast-infiltrating lobular carcinoma	3/13	10/13
Nonmalignant normal thyroid tissue	5/5	0/5	Thyroid papillary carcinoma	0/10	10/10
			Thyroid follicular carcinoma	0/10	10/10
Fibrous tissue	6/6	0/6	Fibrosarcoma	0/10	10/10
Nonmalignant normal bladder tissue	2/2	0/2	Bladder transitional cell carcinoma	0/19	19/19
Nonmalignant normal stomach tissue	0/5	5/5	Stomach adenocarcinoma	0/20	20/20
Cancer adjacent normal kidney tissue	0/5	5/5	Kidney clear-cell carcinoma	0/20	20/20
Cancer adjacent normal brain tissue	0/6	6/6	Cerebrum astrocytoma	2/19	17/19
Cancer adjacent normal ovary tissue	2/6	4/6	Ovary serous papillary carcinoma	0/19	19/19
Cancer adjacent normal liver tissue	0/4	4/4	Hepatocellular carcinoma	0/20	20/20
Cancer adjacent normal pancreas tissue	1/7	6/7	Pancreas adenocarcinoma	4/18	14/18
Cancer adjacent normal testis tissue	0/5	5/5	Testis seminoma	0/20	20/20
Nonmalignant normal lung tissue	0/2	2/2	Lung squamous cell carcinoma and adeno-carcinoma	0/18	18/18
Cancer adjacent normal endometrium tissue	1/5	4/5	Uterus adenocarcinoma endometrium	0/19	19/19
Cancer adjacent normal lymph node tissue	0/9	9/9	Lymph node Hodgkin's disease	0/9	9/9
			B-cell lymphoma	0/9	9/9
Cancer adjacent normal colon tissue	2/4	2/4	Colon adenocarcinoma	1/20	19/20
Cancer adjacent normal prostate tissue	2/7	5/7	Prostate adenocarcinoma	1/18	17/18

Abbreviation: PI4KIIα, phosphatidylinositol 4-kinase type IIα.
The expression level of PI4KIIα in the high-density multiple organ cancer and normal tissue array was analyzed by immunohistochemical staining using an anti-PI4KIIα polyclonal antibody.
[a]Total means total number of cases of each tissue type.

In agreement with these results, a high density of CD31-positive cells and well-organized vasculatures were observed in control tumors. However, in PI4KIIα siRNA HeLa tumors, there were mainly small, truncated blood vessels or single endothelial cells dispersed irregularly in the tumor (Figure 3b). Therefore, the inhibition of tumor growth by the downregulation of PI4KIIα was shown to occur through the suppression of tumor-induced angiogenesis.

As is widely known, angiogenesis depends on endothelial cell survival, migration and formation of new blood vessels (Folkman and Haudenschild, 1980). For this reason, endothelial tube formation assay is frequently adopted to demonstrate angiogenesis *in vitro* (Kubota *et al*., 1988). As shown in Figure 3c, formation of tubes by human umbilical vein endothelial cells (HUVECs) cultured in supernatants from downregulated PI4KIIα HeLa cells was markedly inhibited and the tube length ratio was reduced to ~30% of the control. Consistent with this tube formation result, the 'scratch' wound closure assay showed that HUVECs cultured in supernatants from downregulated PI4KIIα HeLa cells migrated more slowly than did cells in the medium from control HeLa cells (Figure 3d). Both results indicate that the inhibition of PI4KIIα expression in HeLa cells can markedly influence endothelial cell functions, which further confirmed the above result that PI4KIIα can regulate tumor-induced angiogenesis. We then investigated whether suppression of PI4KIIα in tumor cells could influence VEGF protein expression, as VEGF protein expression has been reported to contribute to angiogenesis in tumors (Ferrara, 2002). After knockdown of PI4KIIα, both the secreted VEGF protein in the conditional medium (Figure 3e) and the VEGF protein retained in cancer cells (Figure 3f) were distinctly downregulated. This result confirmed that the expression of PI4KIIα in cancer cells correlates with endothelial cell functions.

Specific regulation of HIF-1α by PI4KIIα in vitro *and* in vivo

As our results showed that both PI4KIIα and HIF-1α are simultaneously upregulated along with tumor growth (Figure 1b), and HIF-1α is a critical transcription factor associated with tumor-induced angiogenesis (Kung *et al*., 2000), the effect of PI4KIIα on HIF-1α expression was investigated in this study. PI4KIIα was downregulated by specific RNA interference in HeLa cells, whereas PI4KIIβ expression was not affected. HIF-1α protein expression was markedly inhibited in PI4KIIα-deficient HeLa cells (Figure 4a) and the effect of PI4KIIα on HIF-1α is also present in MCF-7 cells (Supplementary Figure S4A).

To confirm the direct regulatory effect of PI4KIIα on HIF-1α, pEGFP-PI4kIIα and green fluorescent protein (GFP) were overexpressed in HEK-293 cells as indicated in Figure 4b. The levels of HIF-1α protein were then detected by western blotting with cells transfected with GFP acting as a negative control, whereas cells treated with 1% O_2 for 24 h acted as positive controls (Jiang *et al*., 1997). Results showed that overexpression of

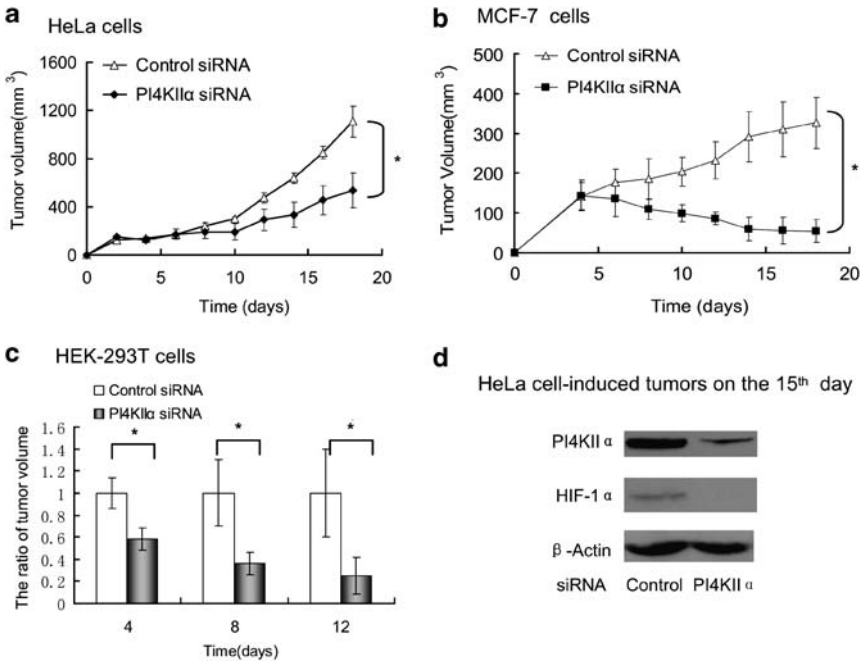

Figure 2 Knockdown of PI4KIIα suppresses tumor growth. BALB/C nude mice (6 mice per group) were subcutaneously injected with 5×10^6 control siRNA HeLa cells or MCF-7 cells or HEK-293T cells in the left side and PI4KIIα siRNA cells in the right side of the venter region. Tumor growth was monitored and is shown as mean volumes ± s.d., $*P<0.05$. (**a**) Effect of PI4KIIα repression on HeLa tumor growth. (**b**) Effect of PI4KIIα repression on MCF-7 tumor growth. (**c**) Effect of PI4KIIα repression on HEK-293T tumor growth. (**d**) After 15 days of transplantation, HeLa cell-induced tumors were harvested and the protein levels of PI4KIIα and HIF-1α were analyzed by western blotting. All figures represent two independent experiments.

PI4KIIα leads to a large accumulation of HIF-1α, while HIF-1α expression was also significantly induced by hypoxia (1% O_2 treated for 24 h). To determine whether the effect of PI4KIIα on HIF-1α is dependent on its kinase activity, the kinase-dead mutant GFP-PI4KIIα (K152A) and its mock vector were overexpressed in HEK-293 cells. As shown, the level of HIF-1α was not affected by the kinase-dead mutant GFP-PI4KIIα (K152A), indicating that the PI4KIIα kinase active site is critical for its effect on HIF-1α (Figure 4b). Furthermore, we examined whether the effect of PI4KIIα siRNA on HIF-1α accumulation can be reversed by direct delivery of PI 4-phosphate (PtdIns(4)P), the production of PtdIns4KIIα. As shown in Supplementary Figure S4 B, the downregulation of HIF-1α protein level induced by PI4KIIα siRNA was obviously rescued by PtdIns(4)P. With regard to the specificity of the regulatory effect of PI4KIIα on HIF-1α, we also studied the effect of PI4KIIβ, another member of the PI4K family, on HIF-1α. As shown in Figure 4b, overexpression of PI4KIIβ did not affect HIF-1α level. We then investigated whether overexpression of PI4KIIα could stimulate the transcriptional activity of HIF-1α by measuring the downstream gene expression of *VEGF* and *iNOS*, both of which were reported to contribute to angiogenesis (Ferrara, 2002; Garcia-Cardena and Folkman, 1998). *VEGF* mRNA and the expression of iNOS protein both were strongly induced by overexpression of PI4KIIα in HeLa cells (Supplementary Figure S5). All these data indicate that the relationship between PI4KIIα and HIF-1α, and the correlation of PI4KIIα with tumor growth, is subtype specific and is dependent on its enzyme activity.

To further determine whether the relationship between PI4KIIα and HIF-1α really exists *in vivo*, we examined the location of both proteins in the same tumor section by using double fluorescence-labeled staining. PI4KIIα in tumor slices yields a stable red fluorescence, which is clearly visible in most cancer cells, whereas HIF-1α yields a green fluorescence, which was only localized in tumor cells with higher levels of red fluorescence (Figure 4c). The same result was obtained in tissues from human breast cancer patients described above (data not shown). These results indicate that HIF-1α localized in cancer cells with high-expressed PI4KIIα, and that HIF-1α is correlated with upregulated PI4KIIα *in vivo*.

PI4KIIα triggers HIF-1α accumulation through a translation-dependent pathway involving the HER-2/PI3K, ERK1/2 cascades

Many reports have concluded that mechanisms responsible for increasing expression of HIF-1α can be divided into two categories, namely increasing protein synthesis and decreasing ubiquitination of HIF-1α (Semenza, 2002; Lee *et al*., 2004). To obtain a better understanding of the processes involved in the increase of HIF-1α protein in response to overexpression of PI4KIIα, we analyzed HIF-1α protein synthesis by performing a time

course of HIF-1α disappearance in the presence of the protein translation inhibitor, cycloheximide. HEK-293 cells were transiently transfected with *GFP-PI4KIIα* or treated with hypoxia (1% O_2) for 24 h, then incubated with cycloheximide for 15, 30 or 60 min. In PI4KIIα-overexpressing HEK-293 cells, cycloheximide led to a decrease in the level of HIF-1α protein after 15 min and HIF-1α protein was no longer detectable after 60 min, but no changes were observed in levels of either overexpressed GFP-PI4KIIα or endogenous PI4KIIα (Figure 5a). In cells exposed to hypoxia, the level of HIF-1α remained constant over a 60-min period (Figure 5b), which was consistent with previous studies showing that hypoxia caused HIF-1α accumulation mainly by blocking its degradation. To confirm whether PI4KIIα can change *HIF-1α* mRNA expression, HEK-293 cells were transfected with *GFP-PI4KIIα* and reverse transcriptase–PCR was performed using a *HIF-1α* cDNA probe (Figure 5c). Just as was the case with hypoxia treatment, overexpression of PI4KIIα did not change the level of *HIF-1α* mRNA. Taken together, these results suggest that PI4KIIα triggers HIF-1α accumulation through a translation-dependent pathway.

According to previous studies, the increase in the synthesis of HIF-1α protein is mainly mediated through the PI3K and mitogen-activated protein kinase pathways (Hellwig-Burgel *et al*., 1999; Zhong *et al*., 2000; Treins *et al*., 2002). Therefore, we examined the effect of inhibitors against PI3K (LY294002) or extracellular signal-regulated protein kinase (ERK)1/2 (U0126) on the accumulation of HIF-1α triggered by overexpression of PI4KIIα. As shown in Figure 5d, both LY294002 and U0126 can reduce the expression of HIF-1α induced by overexpression of PI4KIIα, but neither of them inhibited the induction totally. Genes downstream of HIF-1α, iNOS and *VEGF*, were also analyzed and results were consistent with the above finding: both PI3K and ERK1/2 inhibitors can reduce *VEGF* mRNA and iNOS protein levels triggered by overexpression of PI4KIIα. Moreover, suppression PI4KIIα expression in HeLa cells obviously inhibited the phosphorylation of both ERK1/2 and AKT (Supplementary Figure S6).

As is well known, receptor tyrosine kinase activation is the main factor causing increased HIF-1α synthesis, and functions through the PI3K and mitogen-activated protein kinase pathways (Lee *et al*., 2004); therefore, we hypothesize that PI4KIIα may exert effects on kinases of the epidermal growth factor (EGF)-receptor (also known as ERBB) family, which is a representative subgroup of the receptor tyrosine kinase family. As we have found that suppression of PI4KIIα has the greatest inhibiting effect on breast cancer MCF-7 cell-induced tumor growth, and the expression of PI4KIIα is markedly increased in breast cancer compared with normal breast tissue, the activity of ERBB2 (also known

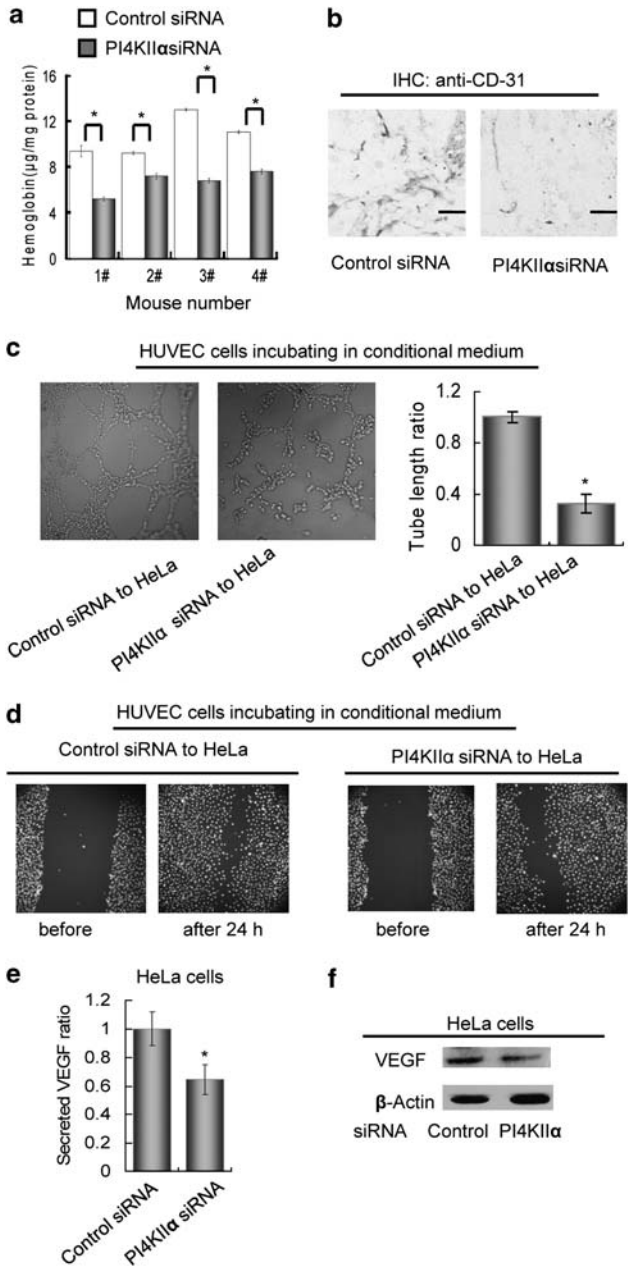

Figure 3 Knockdown of PI4KIIα suppresses tumor-induced angiogenesis, endothelial cell migration, tube formation and the level of the proangiogenesis gene VEGF. (**a**) The hemoglobin content in tumors isolated from mice at day 18, measured by Drabkin's procedure are shown as means ± s.d. (**b**) Cryostat sections of day 18 tumors were prepared and stained with anti-CD31 mAb for blood vessel endothelial cells. The bar represents 100 μm. (**c**) Formation of tubes in HUVECs cultured in supernatants from downregulated PI4KIIα HeLa cells and control cells. Changes in tube length were normalized as the tube length ratio. The culture media of HeLa cells transfected with control siRNA or PI4KIIα siRNA were collected after 72–96 h incubations, and 2×10^4 HUVECs were cultured in either medium in Matrigel-coated wells for 8 h. Micrographs of a typical field were taken for illustration. (**d**) The 'scratch' wound closure assay. Confluent HUVEC monolayers were 'scratch' wounded, and incubated in either of the above HeLa media for 24 h. A typical field is shown to illustrate HUVEC migration. (**e**–**f**) ELISA of VEGF levels in the conditional medium (panel e) and western blotting of VEGF levels in cell lysates (panel f) of PI4KIIα-suppressed and control HeLa cells. Results are shown as means ± s.d., $*P<0.05$. Bar means 100 μm. All figures represent more than two independent experiments.

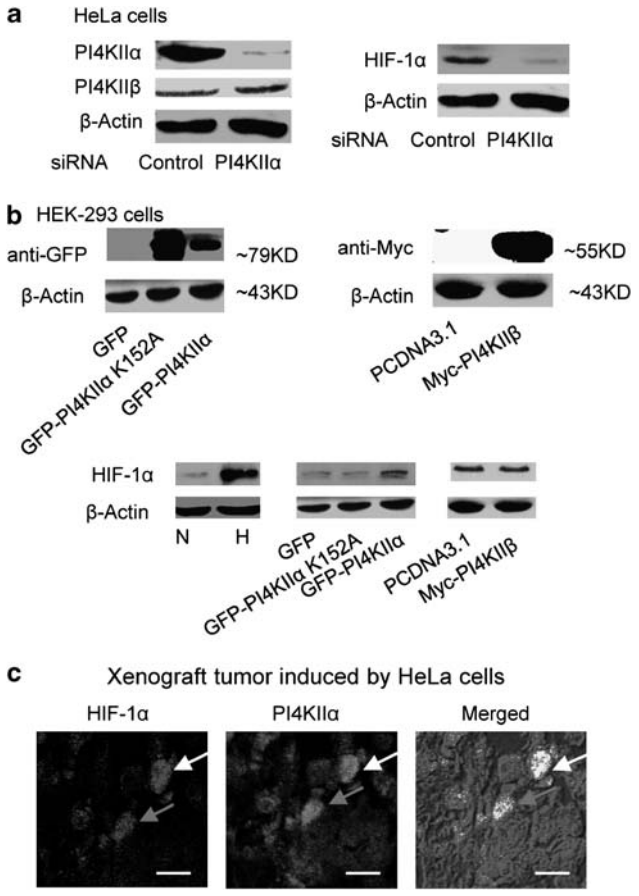

Figure 4 PI4KIIα regulation of HIF-1α. (**a**) The specific effect of PI4KIIα siRNA on protein expression of PI4KIIα analyzed by western blotting in HeLa cells and the effect of downregulation of PI4KIIα on HIF-1α level. (**b**) Overexpression of PI4KIIα, the kinase-dead mutant GFP-PI4KIIα (K152A) or PI4KIIβ analyzed by western blotting in HEK-293 cells and its effects on HIF-1α level. Cell treatment: HEK-293 cells were treated with 21% O_2 for 24 h (N), 1% O_2 for 24 h (H) or transfected with pEGFP-C1, pEGFP-PI4KIIα, pEGFP-PI4KIIαK152A, PCDNA3.1 or PCDNA3.1-myc- PI4KIIβ for 48 h. (**c**) A section of HeLa cell-induced tumor was deparaffinized and stained simultaneously with both rabbit-anti-PI4KIIα and mouse-anti-HIF-1α, and suitable fluorescein-labeled second antibodies were used for detection (red for PI4KIIα and green for HIF-1α). Bar means 10 μm, and the arrowheads with the same color refer to the same cell. All figures represent three independent experiments. A full colour version of this figure is available at the *Oncogene* journal online.

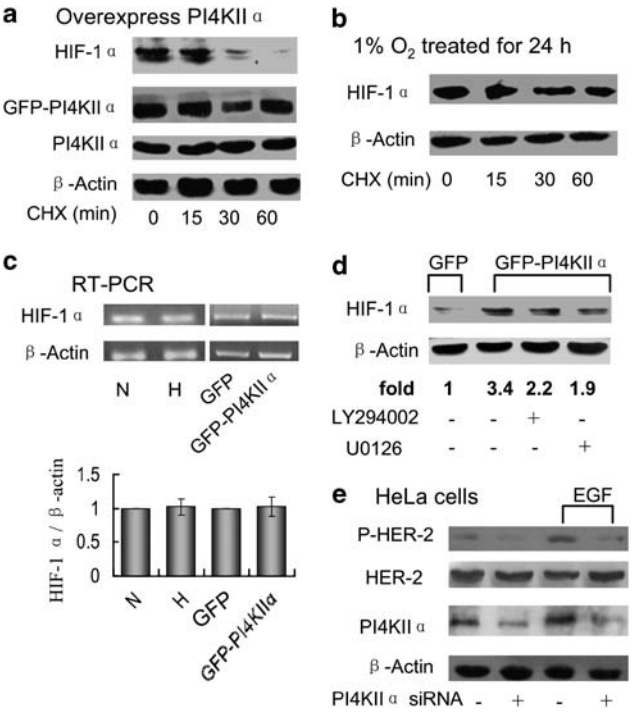

Figure 5 Effects of the protein translation inhibitor cycloheximide (CHX) and inhibitors of PI3K or ERK1/2 on HIF-1α induced by PI4KIIα overexpression. (**a**) Effect of CHX on HIF-1α induced by PI4KIIα overexpression in HEK-293 cells (PI4KIIα and GFP-PI4KIIα protein expression were analyzed in parallel). (**b**) Effect of CHX on HIF-1α protein levels induced by 1% O_2 for 24 h. (**c**) mRNA level of HIF-1α in HEK-293 cells treated as described above was measured and is shown as mean volumes ± s.d. (**d**) Effects of inhibitors of PI3K or ERK1/2 on HIF-1α protein level. After transfection with EGFP-C1 or pEGFP-PI4KIIα for 48 h, HEK-293 cells were treated with or without 10 μM LY294002 (PI3K inhibitor), or 10 μM U0126 (ERK1/2 inhibitor) for 30 min. The band intensity was quantified using Glyko BandScan Version 4.30 (Glyko, Hayward, CA, USA). Changes in protein expression were determined after normalizing the intensity of each band to that of β-actin. (**e**) Effect of suppression of PI4KIIα on HER-2 autophosphorylation at Tyr1248 with or without 100 ng/ml EGF treatment for 20 min in HeLa cells. All figures represent three independent experiments.

as HER-2/neu, which is especially important for breast cancer (Roskoski, 2004) was chosen for examination. As shown in Figure 5e, downregulation of PI4KIIα markedly reduced the autophosphorylation of HER-2 at Tyr1248 under 100 ng/ml EGF stimulation for 20 min, and did not influence its protein level. All the above results indicate that PI4KIIα regulates HIF-1α synthesis through the HER-2/PI3K, ERK pathway.

Discussion

During tumor progression, the expression of many proteins such as HIF-1α, VEGF and HER-2 is upregulated (Ferrara, 2002; Lee *et al*., 2004; Roskoski, 2004), most of which have very important functions in this process. Oncogenes have very important roles in tumor progression. In this study, we found that PI4KIIα has a close relationship with tumor progression: PI4KIIα is significantly upregulated along with tumor growth, and the increased protein levels are strictly localized in cancer cells (Figure 1). What is of greater interest is that specific suppression of PI4KIIα expression can strongly inhibit xenograft tumor growth *in vivo* (Figure 2). On the basis of our research, we propose a model for the mechanism of PI4KIIα in tumor growth: environmental stress causes an increase in PI4KIIα protein levels in cancer cells, and the elevated PI4KIIα levels lead to increased HER-2 activities, which can increase HIF-1α protein accumulation in the PI3K- and ERK1/2-dependent pathways. The increased HIF-1α activates the expression of its proangiogenesis target

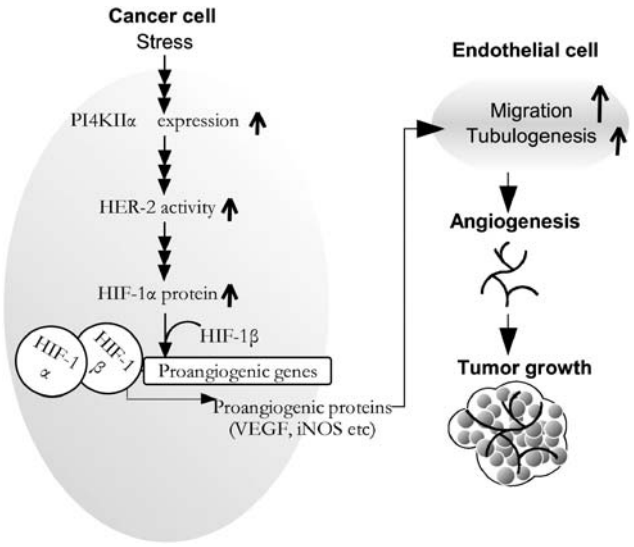

Figure 6 A model for the mechanism of PI4KIIα action in tumor growth. Environmental stress causes an increase in PI4KIIα protein levels, and the elevated PI4KIIα leads to increased HER-2 activities, which can increase HIF-1α protein accumulation in the PI3K- and ERK1/2-dependent pathways. The accumulated HIF-1α can heterodimerize with HIF-1β to activate the transcription of downstream proangiogenic genes, such as VEGF and iNOS, which can promote endothelial cell migration and tubulogenesis. As a result, tumor growth is promoted by the enhanced angiogenesis. Knockdown of PI4KIIα significantly inhibits this pathway and represses tumor growth.

genes, such as *VEGF* and *iNOS*, to promote endothelial cell migration and tubulogenesis, contributing to tumor angiogenesis. Knockdown of PI4KIIα in tumor cells significantly inhibits this pathway and suppresses tumor growth (Figure 6). To our knowledge, this is the first report to suggest that PI4KIIα is crucial for the regulation of tumor-induced angiogenesis through HIF-1α.

With respect to the role of PI4K in cancer, a previous study simply reported that most PI kinases have higher activities in cancer cells (Weber *et al*., 1996). In this study, we determined that it is the protein level of one specific subtype, PI4KIIα, that increases in several types of human cancer and that the upregulation of PI4KIIα directly contributes to tumor progression. Results from our tumor microarray study strongly indicate that 95% of malignant melanomas (dermis), 88.9% of human breast nonspecific infiltrating duct carcinomas, 76.9% of breast infiltrating lobular carcinomas, nearly 100% of thyroid papillary carcinomas, 100% of thyroid follicular carcinomas, 100% of fibrosarcomas and 100% of bladder transitional cell carcinomas have elevated PI4KIIα expression levels compared with normal tissues. However, we were not able to conclude whether PI4KIIα expression also changes in the other 13 types of cancer tested (Table 1), as all the samples were displayed on the same chip—it is hard to control staining conditions so that they are suitable for each sample. These results show that these 13 types of cancer all have high levels of PI4KIIα expression in both cancer and normal samples. In this study, three different tumor cell lines (namely HeLa, MCF-7 and HEK-293) were used to test the effects of PI4KIIα on tumor growth. Downregulation of PI4KIIα resulted in a clear inhibition of tumor growth in all these cell lines, particularly in MCF-7 cell-induced tumors, in which tumor growth was almost completely inhibited (Figure 2b). Taken together with results obtained from the human tumor microarray, we can conclude that PI4KIIα is an effective target for tumor control in more than one tumor type. However, its efficacy may vary with different tumor types. One point to be addressed is that both overexpressing PI4KIIα and its kinase-dead mutant PI4KIIαk152A in HeLa cells had no significant effect on the growth of tumor transplants (Supplementary Figure S7). The reason for this might be that the upregulation of endogenous PI4KIIα on tumor growth is so strong that the effects of overexpressed exogenous PI4KIIα are largely submerged. A very recent study showed that inositol polyphosphate 4-phosphatase type II (INPP4B) is a tumor suppressor (Gewinner *et al*., 2009). Several aspects of this study were consistent with our work, for example, the overexpression of INPP4B in SUM 149 cells reduced tumor growth in a xenograft mouse model by suppressing the PI3K/AKT signaling pathway, and INPP4B is frequently deleted in various solid tumors, including basal-like breast cancer. As known, the relationship between PI4K and INPP4B is similar to PI3K and PTEN; thus, both studies suggested that the D-4 position phosphorylated productions of PI are essential for tumor growth. In this regard, the regulation of D-4 position phosphorylation of PIs is a multi-mechanism in tumor progression.

With respect to the regulation of the HER-2 signaling pathway by PI4KIIα, there have been some reports about the relationship between PI4KIIα and different ERBB families: Scott *et al*. (1991) showed that PI4K activity increased after stimulation of the ERBB-2 (HER-2) receptor tyrosine kinase in overexpressing breast cancer cells (Scott *et al*., 1991). Kauffmann-Zeh *et al*. (1994) reported that EGF regulates PI4KIIα activity and is associated with ERBB-1 (EGFR), and Minogue *et al*. (2006) showed that PI4KIIα can influence the endosomal trafficking and degradation of EGFR. However, none of these reports show a direct effect of PI4KIIα on kinase activity. In this study, we report that regulation of PI4KIIα expression can markedly influence the autophosphorylation of HER-2 at Tyr1248, which may impact members of the signaling pathway downstream of HER-2, such as PI3K and ERK, therefore contributing to the accumulation of HIF-1α, a very important molecule for tumor-induced angiogenesis (Figure 5). It was also interesting to discover that the effect of PI4KIIα on HIF-1α is subtype specific and kinase activity dependent (Figure 4), similar to the effects of PI4KIIα on the Wnt signaling pathway and on adaptor complex-1 and adaptor complex-3 functions (Wang *et al*., 2003; Craige *et al*., 2008; Pan *et al*., 2008). Although we are still not clear about the detailed mechanism, we hypothesize that the effect of PI4KIIα on the HER-2 signal transduction cascade may

be through a similar mechanism to that for its effect on the Wnt signal pathway: suppression of PI4KIIα may influence the formation of PtdIns(4)P and PtdIns(4,5)P_2 in the plasma membrane, which are essential for HER-2 heterodimerization with other members of the ERBB family, such as EGFR, HER-3 and HER-4. Further investigation of this mechanism will be of value in future studies.

With respect to the function of PI4KIIα in angiogenesis, we found that supernatants from downregulated PI4KIIα HeLa cells significantly inhibited endothelial migration and tube formation. What is more, the proangiogenic gene VEGF produced by HeLa cells both in supernatants and cell lysates was dramatically reduced after PI4KIIα knockdown (Figure 3). Further study indicated that PI4KIIα can directly influence the accumulation of HIF-1α (Figure 4a and b), an important regulator of VEGF in tumor progression (Huang and Bunn, 2003). Moreover, we found that HIF-1α was mainly located in cancer cells overexpressing PI4KIIα (Figure 4c), in accordance with *in vitro* observations, confirming the correlation between PI4KIIα and HIF-1α *in vivo*. All these results provide a good explanation for the effect of PI4KIIα on angiogenesis. Therefore, PI4KIIα is a novel player in tumor control through the regulation of angiogenesis.

To our knowledge, this is the first time that the pivotal role of PI4KIIα in the regulation of tumor growth through angiogenesis and HIF-1α has been demonstrated in its own right. Our findings indicate that PI4KIIα is a new player in the orchestration of tumor control. In addition, PI4KIIα is just one subtype of the PI4K family; thus, specific downregulation of PI4KIIα will not induce complicated side effects because other PI4K family members remain functional. Therefore, PI4KIIα may be a promising target for tumor therapy.

Materials and methods

Human cancer specimens

A multiple organ cancer tissue and normal tissue microarray was purchased from Chaoying Biotechnology Company (Shanxi, China). Patient breast cancer tissues and the corresponding cancer adjacent normal tissues were obtained from Xuanwu Hospital (Capital Medical University, Beijing, China). All patients consented to the use of their tissues for research projects.

Analysis of hemoglobin content in tumors

Hemoglobin content in isolated tumors was estimated by Drabkin's cyanmethemoglobin method (Drabkin, 1949). Hemoglobin values were calculated using a hemoglobin standard curve prepared using serial dilutions of mouse blood.

Endothelial cell scratch wound healing assay and tube formation assay

HeLa cells were transfected with control siRNA or PI4KIIα siRNA as described above. After a 72-h incubation period, the culture medium was changed with fresh medium containing 10% fetal bovine serum, and the supernatant from both types of transfected cells was collected after a further 24-h incubation period. Confluent HUVEC monolayers were 'scratch' wounded and then rinsed with phosphate-buffered saline. The above supernatants were then added separately to the wells. After incubation for 24 h, cells were stained with Hoechst 33342, and then observed on a laser-scanning confocal microscope (Olympus FV500, Olympus, Tokyo, Japan). Three measurements taken randomly were obtained for each treatment and the experiment was repeated three times. The endothelial tube formation assay was performed as described previously with the following modifications (Grant *et al*., 1992). HUVECs were cultured in either of the HeLa cell media described above. The HUVEC tube length was analyzed using Scion Image analysis software (Scion, Frederick, MD, USA). Changes in tube length were normalized as the tube length ratio.

Animal studies

Six-to-eight-week old male BALB/c nude mice (purchased from Weitonglihua, Beijing, China) were allowed to acclimatize for 1 week under specific pathogen-free conditions in the animal facility of the Institute of Biophysics, Chinese Academy of Sciences. All procedures involving animals and their care were approved by the corresponding authorities. In all, 200 μl suspensions of treated cells (HeLa cells, MCA205 tumor cells or MCF-7 cells, phosphate-buffered saline; 2.5×10^7 cells per ml) and their controls were subcutaneously injected into BALB/c nude mice at each venter region and tumor growth was monitored every 2 days. The tumor volumes were calculated (Tan *et al*., 2004) and tumor tissues were isolated on the days indicated for the measurement of PI4KIIα and HIF-1α protein levels by western blotting and immunohistochemical analysis.

Statistical analysis

Statistical analyses were performed using the two-tailed paired Student *t*-test. We considered data statistically significant when the *P*-value was <0.05. All data are expressed as means ± s.d.

Conflict of interest

A patent has been applied by the Institute of Biophysics, CAS, CHINA (CC, JML).

Acknowledgements

We thank Shane Minogue and Konstantin V Kandror for gifted plasmids and Pietro De Camilli for PI4KIIα antibody, Wei Liang, Xiyun Yan and Yi Zhu for sharing materials, and Guoheng Xu and Qinwei Yin for valuable discussions. We are grateful to Nanping Wang for his great support. We also thank Junfeng Hao and Xudong Zhao for technical assistance. The work was supported by the '863' National High-Technology Development Program of China (0A200202D03), the National Basic Research Program of China (2006CB911001, 2005CB522804) and the National Natural Science Foundation of China (90606020, 30770512).

References

Craige B, Salazar G, Faundez V. (2008). Phosphatidylinositol-4-kinase type II alpha contains an AP-3-sorting motif and a kinase domain that are both required for endosome traffic. *Mol Biol Cell* **19**: 1415–1426.

Di PG, De CP. (2006). Phosphoinositides in cell regulation and membrane dynamics. *Nature* **443**: 651–657.

Drabkin D. (1949). The standardization of hemoglobin measurement. *Am J Med Sci* **217**: 710–711.

Ferrara N. (2002). VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* **2**: 795–803.

Folkman J. (1971). Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**: 1182–1186.

Folkman J, Haudenschild C. (1980). Angiogenesis *in vitro*. *Nature* **288**: 551–556.

Garcia-Cardena G, Folkman J. (1998). Is there a role for nitric oxide in tumor angiogenesis? *J Natl Cancer Inst* **90**: 560–561.

Gehrmann T, Heilmeyer Jr LM. (1998). Phosphatidylinositol 4-kinases. *Eur J Biochem* **253**: 357–370.

Gewinner C, Wang ZC, Richardson A, Teruya-Feldstein J, Etemadmoghadam D, Bowtell D *et al*. (2009). Evidence that inositol polyphosphate 4-phosphatase type II is a tumor suppressor that inhibits PI3K signaling. *Cancer Cell* **16**: 115–125.

Grant DS, Kinsella JL, Fridman R, Auerbach R, Piasecki BA, Yamada Y *et al*. (1992). Interaction of endothelial cells with a laminin A chain peptide (SIKVAV) *in vitro* and induction of angiogenic behavior *in vivo*. *J Cell Physiol* **153**: 614–625.

Guo J, Wenk MR, Pellegrini L, Onofri F, Benfenati F, De CP. (2003). Phosphatidylinositol 4-kinase type IIalpha is responsible for the phosphatidylinositol 4-kinase activity associated with synaptic vesicles. *Proc Natl Acad Sci USA* **100**: 3995–4000.

Hanahan D, Folkman J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**: 353–364.

Heilmeyer Jr LM, Vereb Jr G, Vereb G, Kakuk A, Szivak I. (2003). Mammalian phosphatidylinositol 4-kinases. *IUBMB Life* **55**: 59–65.

Hellwig-Burgel T, Rutkowski K, Metzen E, Fandrey J, Jelkmann W. (1999). Interleukin-1beta and tumor necrosis factor-alpha stimulate DNA binding of hypoxia-inducible factor-1. *Blood* **94**: 1561–1567.

Hsuan JJ. (1993). Oncogene regulation by growth factors. *Anticancer Res* **13**: 2521–2532.

Huang LE, Bunn HF. (2003). Hypoxia-inducible factor and its biomedical relevance. *J Biol Chem* **278**: 19575–19578.

Jiang BH, Zheng JZ, Leung SW, Roe R, Semenza GL. (1997). Transactivation and inhibitory domains of hypoxia-inducible factor 1alpha. Modulation of transcriptional activity by oxygen tension. *J Biol Chem* **272**: 19253–19260.

Kauffmann-Zeh A, Klinger R, Kauffmann-Zeh A, Endemann G, Waterfield MD, Wetzker R *et al*. (1994). Regulation of human type II phosphatidylinositol kinase activity by epidermal growth factor-dependent phosphorylation and receptor association. *J Biol Chem* **269**: 31243–31251.

Koesters R, von Knebel DM. (2003). The Wnt signaling pathway in solid childhood tumors. *Cancer Lett* **198**: 123–138.

Kubota Y, Kleinman HK, Martin GR, Lawley TJ. (1988). Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. *J Cell Biol* **107**: 1589–1598.

Kung AL, Wang S, Klco JM, Kaelin WG, Livingston DM. (2000). Suppression of tumor growth through disruption of hypoxia-inducible transcription. *Nat Med* **6**: 1335–1340.

Lee JW, Bae SH, Jeong JW, Kim SH, Kim KW. (2004). Hypoxia-inducible factor (HIF-1)alpha: its protein stability and biological functions. *Exp Mol Med* **36**: 1–12.

Melillo G, Musso T, Sica A, Taylor LS, Cox GW, Varesio L. (1995). A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *J Exp Med* **182**: 1683–1693.

Minogue S, Anderson JS, Waugh MG, dos Santos M, Corless S, Cramer R *et al*. (2001). Cloning of a human type ii phosphatidylinositol 4-kinase reveals a novel lipid kinase family. *J Biol Chem* **276**: 16635–16640.

Minogue S, Waugh MG, De Matteis MA, Stephens DJ, Berditchevski F, Hsuan JJ. (2006). Phosphatidylinositol 4-kinase is required for endosomal trafficking and degradation of the EGF receptor. *J Cell Sci* **119**: 571–581.

Pan W, Choi SC, Wang H, Qin Y, Volpicelli-Daley L, Swan L *et al*. (2008). Wnt3a-mediated formation of phosphatidylinositol 4,5-bisphosphate regulates LRP6 phosphorylation. *Science* **321**: 1350–1353.

Qin Y, Li L, Pan W, Wu D. (2009). Regulation of phosphatidylinositol kinases and metabolism by Wnt3a and Dvl2. *J Biol Chem* **284**: 22544–22548.

Roskoski Jr R. (2004). The ErbB/HER receptor protein-tyrosine kinases and cancer. *Biochem Biophys Res Commun* **319**: 1–11.

Salazar G, Zlatic S, Craige B, Peden AA, Pohl J, Faundez V. (2009). Hermansky-Pudlak syndrome protein complexes associate with phosphatidylinositol 4-kinase type II alpha in neuronal and non-neuronal cells. *J Biol Chem* **284**: 1790–1802.

Scott GK, Dodson JM, Montgomery PA, Johnson RM, Sarup JC, Wong WL *et al*. (1991). p185HER2 signal transduction in breast cancer cells. *J Biol Chem* **266**: 14300–14305.

Semenza GL. (2001). Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med* **7**: 345–350.

Semenza GL. (2002). HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med* **8**: S62–S67.

Simons JP, Al-Shawi R, Minogue S, Waugh MG, Wiedemann C, Evangelou S *et al*. (2009). Loss of phosphatidylinositol 4-kinase 2alpha activity causes late onset degeneration of spinal cord axons. *Proc Natl Acad Sci USA* **106**: 11535–11539.

Tan C, Cruet-Hennequart S, Troussard A, Fazli L, Costello P, Sutton K *et al*. (2004). Regulation of tumor angiogenesis by integrin-linked kinase (ILK). *Cancer Cell* **5**: 79–90.

Treins C, Giorgetti-Peraldi S, Murdaca J, Semenza GL, Van OE. (2002). Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *J Biol Chem* **277**: 27975–27981.

Wang YJ, Wang J, Sun HQ, Martinez M, Sun YX, Macia E *et al*. (2003). Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. *Cell* **114**: 299–310.

Waugh MG, Minogue S, Anderson JS, Balinger A, Blumenkrantz D, Calnan DP *et al*. (2003). Localization of a highly active pool of type II phosphatidylinositol 4-kinase in a p97/valosin-containing-protein-rich fraction of the endoplasmic reticulum. *Biochem J* **373**: 57–63.

Weber G, Shen F, Prajda N, Yeh YA, Yang H, Herenyiova M *et al*. (1996). Increased signal transduction activity and down-regulation in human cancer cells. *Anticancer Res* **16**: 3271–3282.

Xu Z, Huang G, Kandror KV. (2006). Phosphatidylinositol 4-kinase type IIalpha is targeted specifically to cellugyrin-positive glucose transporter 4 vesicles. *Mol Endocrinol* **20**: 2890–2897.

Yamamoto S, Tomita Y, Hoshida Y, Iizuka N, Monden M, Yamamoto S *et al*. (2004). Expression level of valosin-containing protein (p97) is correlated with progression and prognosis of non-small-cell lung carcinoma. *Ann Surg Oncol* **11**: 697–704.

Zhong H, Chiles K, Feldser D, Laughner E, Hanrahan C, Georgescu MM *et al*. (2000). Modulation of hypoxia-inducible factor 1alpha expression by the epidermal growth factor/phosphatidylinositol 3-kinase/PTEN/AKT/FRAP pathway in human prostate cancer cells: implications for tumor angiogenesis and therapeutics. *Cancer Res* **60**: 1541–1545.

Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)