VEGF-mediated PI3K class IA and PKC signaling in cardiomyogenesis and vasculogenesis of mouse embryonic stem cells

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Summary

VEGF-, phosphoinositide 3-kinase (PI3K)- and protein kinase C (PKC)-regulated signaling in cardiac and vascular differentiation was investigated in mouse ES cells and in ES cell-derived Flk-1$^+$ cardiovascular progenitor cells. Inhibition of PI3K by wortmannin and LY294002, disruption of PI3K catalytic subunits p110α and p110δ using short hairpin RNA (shRNA), or inhibition of p110α with compound 15e and of p110δ with IC-87114 impaired cardiac and vascular differentiation. By contrast, TGX-221, an inhibitor of p110β, and shRNA knockdown of p110β were without significant effects. Antagonists of the PKC family, i.e. bisindolylmaleimide-1 (BIM-1), GÖ 6976 (targeting PKCα/βII) and rottlerin (targeting PKCδ) abolished vasculogenesis, but not cardiomyogenesis. Inhibition of Akt blunted cardiac as well as vascular differentiation. VEGF induced phosphorylation of PKCα/βII and PKCδ but not PKCζ. This was abolished by PI3K inhibitors and the VEGFR-2 antagonist SU5614. Furthermore, phosphorylation of Akt and phosphoinositide-dependent kinase-1 (PDK1) was blunted upon inhibition of PI3K, but not upon inhibition of PKC by BIM-1, suggesting that activation of Akt and PDK1 by VEGF required PI3K but not PKC. In summary, we demonstrate that PI3K catalytic subunits p110α and p110δ are central to cardiovasculogenesis of ES cells. Akt downstream of PI3K is involved in both cardiomyogenesis and vasculogenesis, whereas PKC is involved only in vasculogenesis.

Key words: Embryonic stem cells, Cardiovascular differentiation, Phosphoinositide 3-kinase, Protein kinase C

Introduction

The dissection of signaling pathways regulating cardiac versus vascular differentiation is of particular impact because recent evidence has shown that, during embryogenesis, cardiomyogenesis and vasculogenesis occur in a strictly time- and space-dependent manner. Using mouse embryonic stem (ES) cells, which mimic early embryonic development, it was discovered that cardiac-independent vasculogenesis arises from Flk-1$^+$ (vascular endothelial growth factor receptor 2-positive, VEGFR2$^+$) hemangioblasts, whereas at cardiogenic day 3.25 a 'second' Flk-1$^+$ cell population with enriched cardiogenic as well as vasculogenic potential was identified (Yang et al., 2008). This observation raised the intriguing possibility that the cardiac lineage develops from a progenitor cell that also displays vascular potential and thus might represent the cardiac equivalent to the hemangioblast (Bu et al., 2009; Garry and Olson, 2006; Kattman et al., 2007). As a consequence, Flk-1$^+$ cardiac as well as endothelial progenitor cells should be responsive towards VEGF stimulation, and might activate distinct signaling pathways that variegate cardiac-associated vasculogenesis versus cardiac-independent (yolk sac hemangioblast-derived) vascular differentiation (Iida et al., 2005; Kattman et al., 2006).

The signal transduction pathways that are activated in different cell types in response to VEGF are well established (Cross et al., 2003; Wang et al., 2008). However, relatively little is known about the involvement of distinct phosphoinositide 3-kinase (PI3K) catalytic subunits and protein kinase C (PKC) isoforms in VEGF action (Hamada et al., 2005; Gliki et al., 2002; Gerber et al., 1998). VEGF receptors (VEGFR1 and VEGFR2) are present on vascular endothelial cells and their signaling is mediated by receptor dimerization leading to autophosphorylation of the cytosolic domains of the receptors. Phosphorylated VEGF receptors serve as docking sites for adapter molecules or signaling enzymes such as PI3K. VEGF has been shown to activate PI3K generating phosphatidylinositol (3,4,5)-trisphosphates (Bos 1995; Gerber et al., 1998). Moreover, numerous studies demonstrated that PI3K plays an important role in regulating endothelial proliferation, migration and survival (Gerber et al., 1998; Thakker et al., 1999; Jiang et al., 2000).

PI3Ks are classified into classes I, II and III (Vanhaesebroeck et al., 1997). Class I PI3Ks, which include class IA and class IB (consisting of PI3Kγ only), are a family of dual-specificity lipid and protein kinases that control many cellular functions, such as growth and proliferation, survival and apoptosis, as well as adhesion and migration of a wide range of cell types (Katso et al., 2001; Wymann and Pirola, 1998). All four class I PI3Ks are heterodimers composed of a catalytic subunit with a molecular weight of 110 kDa and a tightly associated regulatory subunit that controls activation and subcellular localization (Whitman et al., 1988; Stephens et al., 1991). The importance of PKC regarding the VEGF–PI3K pathway was suggested by several authors (Chou et al., 1998; Dutil et al., 1998; Gliki et al., 2002; Le Good et al., 1998), although a detailed VEGF-

related mechanism involving PKC has not been determined until now in differentiating ES cells. PKC is a family of protein kinases and phospholipid-dependent enzymes that consists of at least of 11 isozymes (Teicher, 2006). They are classified as conventional (α, β1, β2, γ), novel (δ, ε, η, θ, μ) and atypical (ζ, λ) isozymes.

The aim of this study was to identify the role of PI3K class IA (consisting of PI3Kα, PI3Kβ and PI3Kδ isoforms containing p110α, p110β and p110δ catalytic subunits, respectively) and their downstream effector pathways, i.e. PKC (PKCα/βII, PKCδ and PKCζ), in signaling pathways that diversify cardiomyogenesis versus vasculogenesis, and elucidate the impact of VEGF in these processes. Our data demonstrate that PI3K is a key regulator of cardiovascular differentiation of ES cells, and outline a specific role of the PI3K–Akt pathway in cardiomyogenesis and vasculogenesis versus a PI3K–PDK1–PKC pathway regulating vasculogenesis independently of cardiomyogenesis.

Results

Pharmacological inhibition of PI3Ks and targeting of class IA p110 catalytic subunits affects cardiac and vascular differentiation of ES cells

In the present study, the necessity of VEGF-regulated PI3K- and PKC-signaling pathways for the process of cardiomyogenesis as well as vasculogenesis was investigated in embryoid bodies (EBs) derived from CGR8 ES cells. These pathways and subsequent catalytic subunits are expressed in parallel to the first steps of cardiovascular differentiation (supplementary material Fig. S1A,B; *n*=3). To examine the effect of PI3K inhibition on cardiovascular differentiation, EBs were incubated with the general PI3K inhibitors wortmannin (1 μM) and LY294002 (50 μM) in the presence or absence of VEGF from day 4 to day 10 of cell culture. In parallel, specific inhibitors of distinct class 1A PI3K catalytic subunits, i.e. the p110α inhibitor compound 15e (0.5 μM), the p110β inhibitor TGX-221 (1 μM) and the p110δ inhibitor IC-87114 (1 μM), were applied in the presence or absence of VEGF (Fig. 1A–E; *n*=5). None of the pharmacological inhibitors significantly activated cleaved caspase 3 as a signature of apoptosis (supplementary material Fig. S2A,B; *n*=3). However, protein expression of the proliferation marker Ki-67 was significantly decreased in EBs treated with wortmannin, LY297002, compound 15e and IC-87114, but not with TGX-221 (supplementary material Fig. S2C; *n*=3), which resulted in decreased EB growth. Treatment of EBs with VEGF significantly increased CD31- and α-actinin-positive cell areas (Fig. 1A–C). Incubation of EBs with wortmannin and LY294002 significantly reduced the size of cardiac areas (Fig.

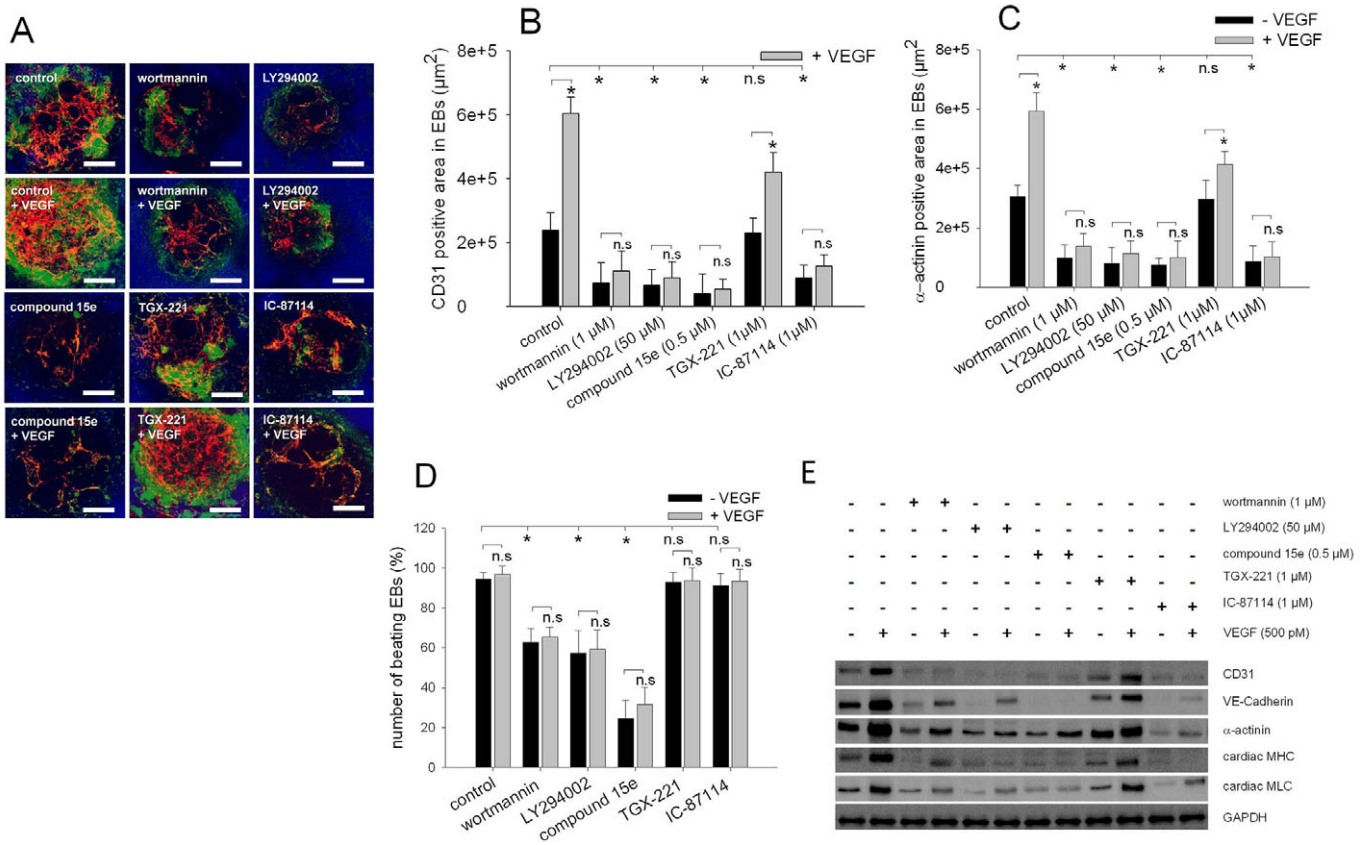

Fig. 1. Effect of general and specific PI3K class 1A catalytic subunit inhibitors on cardiovascular differentiation. (**A**) Representative immunohistochemistry (IHC) images analyzing the effect of general [Wortmannin (1 μM), LY294002 (50 μM)], and specific PI3K class 1A catalytic subunit inhibitors [compound 15e (0.5 μM), TGX-221 (1 μM) and IC-87114 (1 μM)] regarding cardiovascular differentiation of EBs. The substances were applied from day 4 to day 10 of cell culture either in the absence or presence of VEGF (500 pM). Shown are representative EBS immunostained with antibodies directed against CD31 (red) or α-actinin (green). Scale bars: 100 μm. (**B**,**C**) Semiquantitative analysis of CD31-positive vascular (see B) and α-actinin-positive (see C) cell areas. (**D**) Analysis of the percentage of beating EBs upon inhibition of PI3K. (**E**) Western blot analysis of endothelial (VE-Cadherin and CD31) and cardiac-specific makers (α-actinin, cardiac MHC, cardiac MLC) upon treatment with PI3K inhibitors. GAPDH was used as endogenous control. All analyses were done on day 10 of cell culture. **P*<0.05, as indicated.

1A,C) and CD31-positive vascular areas (Fig. 1A,B) as well as the protein expression of CD31 and VE-Cadherin in the treated EBs (Fig. 1E). Strongest effects were achieved upon treatment with compound 15e (0.5 μM), which is a specific PI3K p110α inhibitor. Moreover, the percentage of beating areas per EB (Fig. 1D) and the protein expression of typical cardiac genes (α-actinin, cardiac MHC, cardiac MLC) (Fig. 1E) were significantly decreased upon incubation with wortmannin, LY294002 and compound 15e. Treatment with IC-87114 (1 μM), an inhibitor of the PI3K p110δ subunit, only reduced the size of cardiac and vascular areas (see Fig. 1A–C) as well as protein expression (see Fig. 1E), whereas the percentage of beating EBs remained unaffected (see Fig. 1D). Finally, treatment with the p110β inhibitor TGX-221 (1 μM) did not exert significant effects on cardiovascular cell area, typical cardiovascular proteins and the percentage of spontaneously contracting EBs (see Fig. 1A–E). A potential lineage shift towards the neuronal lineage caused by an inhibition of PI3K isoforms was excluded because mRNA expression of Nestin and neuron-specific enolase (NSE) was not changed upon treatment with PI3K inhibitors (data not shown). It was apparent that all inhibitors of PI3K downregulated the expression of cardiovascular genes, with most pronounced effects observed upon inhibition with the PI3K p110α inhibitor compound 15e and weakest effects with the p110β inhibitor TGX-221. None of the described effects was abolished through parallel application of VEGF.

Silencing of PI3Ks class IA p110 catalytic subunits using shRNA technology

To verify the results obtained from the pharmacological inhibition we used stable short hairpin RNA (shRNA) transduction targeting PI3K p110 catalytic subunits. A specific and significant inhibition of class IA PI3K isoforms was detected in clone V for P110α subunit (supplementary material Fig. S3A), in clone I for p110β subunit (supplementary material Fig. S3B), and in clone IV for p110δ subunit (supplementary material Fig. S3C). Interestingly,

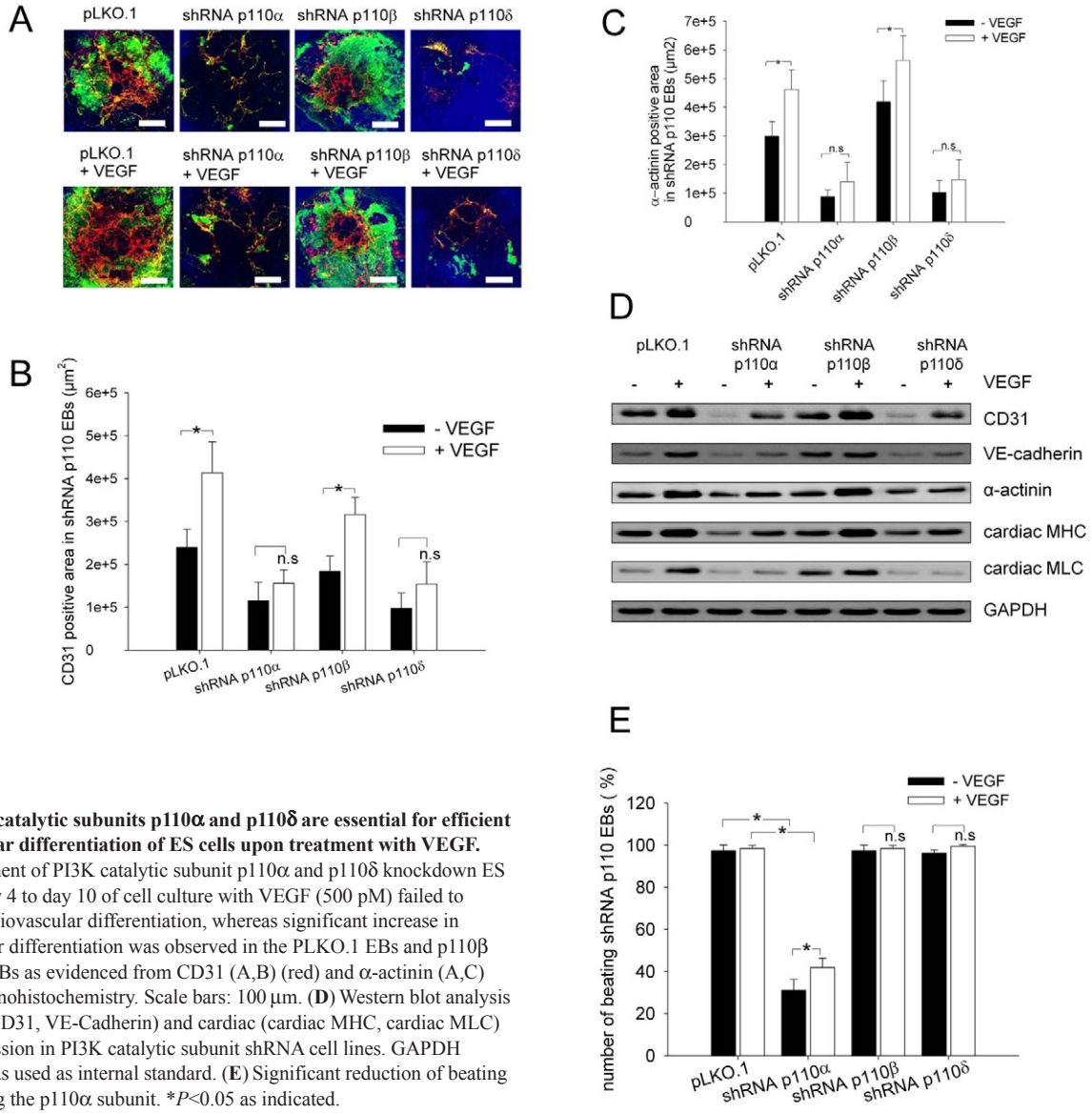

Fig. 2. PI3K catalytic subunits p110α and p110δ are essential for efficient cardiovascular differentiation of ES cells upon treatment with VEGF. (**A–C**) Treatment of PI3K catalytic subunit p110α and p110δ knockdown ES cells from day 4 to day 10 of cell culture with VEGF (500 pM) failed to stimulate cardiovascular differentiation, whereas significant increase in cardiovascular differentiation was observed in the PLKO.1 EBs and p110β knockdown EBs as evidenced from CD31 (A,B) (red) and α-actinin (A,C) (green) immunohistochemistry. Scale bars: 100 μm. (**D**) Western blot analysis of vascular (CD31, VE-Cadherin) and cardiac (cardiac MHC, cardiac MLC) marker expression in PI3K catalytic subunit shRNA cell lines. GAPDH expression was used as internal standard. (**E**) Significant reduction of beating in EBs lacking the p110α subunit. *P<0.05 as indicated.

silencing of the PI3K p110β subunit resulted in complementary increase of p110α subunit expression in shRNA p110β (see supplementary material Fig. S3B). In corroboration with the results achieved upon pharmacological inhibition of p110α with compound 15e, mRNA silencing of p110α resulted in the most pronounced inhibition of cardiovascular differentiation. Downregulation of p110δ resulted in significant decrease in the size of cardiac and vascular cell areas, whereas the percentage of spontaneously contracting EBs was not affected (supplementary material Fig. S4A,B). By contrast, a non-significant decrease in vascular areas and significantly increased cardiac areas were observed after silencing p110β (see supplementary material Fig. S4A), whereas the number of spontaneously contracting EBs was not affected (supplementary material Fig. S4B). This might be due to the upregulation of p110α achieved upon downregulation of p110β (see supplementary material Fig. S3B).

PI3K catalytic subunit p110α and p110δ are essential for cardiovascular differentiation upon treatment of EBs with VEGF

As previously described, VEGF plays a crucial role in cardiac and vascular differentiation, and acts on Flk-1^{+} cardiovascular progenitor cells (Chen et al., 2006; Cheung, 1997; Gerber et al., 1998; Gliki et al., 2002; Song et al., 2007; Yang et al., 2002; Zisa et al., 2009; Lange et al., 2009). To investigate whether PI3K class IA isoforms are essential for VEGF signaling pathways leading to cardiac and vascular differentiation of ES cells, p110α, p110β and p110δ gene-inactivated EBs were treated from day 4 to day 10 of cell culture with VEGF (500 pM). This treatment resulted in a significant increase in cardiovascular differentiation in the control pLKO.1 EBs and in p110β knockout EBs, as evaluated by quantification of cardiac and vascular cell areas (Fig. 2A–C; *n*=3) and western blot analysis (Fig. 2D; *n*=3). By contrast, stimulation of cardiovascular differentiation upon treatment with VEGF was nearly absent in EBs lacking p110α and p110δ subunits (see Fig. 2A–C). When the percentage of spontaneously contracting EBs was assessed, it was apparent that silencing of PI3K p110α subunit resulted in significant reduction of beating EBs down to 40% in comparison with control EBs, which could not be restored upon treatment with VEGF (Fig. 2E; *n*=3). Taken together, our experiments demonstrate that the PI3K catalytic subunits p110α and p110δ regulate cardiac and vascular differentiation of EBs derived from murine ES cells and are involved in VEGF-mediated cardiovascular signaling pathways.

PKC inhibition blunts vasculogenesis independent of cardiomyogenesis

The importance of PKC in the VEGF–PI3K pathway was suggested by several authors (Chou et al., 1998; Dutil et al., 1998; Gliki et al., 2002; Le Good et al., 1998), but the actual mechanisms of PKC stimulation by VEGF are not known. To evaluate the role of PKC during cardiovascular differentiation of ES cells, EBs were treated from day 4 to day 10 of cell culture with inhibitors of the PKC family, i.e. bisindolylmaleimide-1 (BIM-1), GÖ 6976 (inhibitor of PKC α/βII), rottlerin (inhibitor of PKCδ) and myristoylated PKCζ peptide (myr-PKCζ peptide). Immunohistochemistry (Fig. 3A,B; *n*=4) and western blot (Fig. 3C; *n*=4) showed that vasculogenesis was decreased in EBs preincubated with PKC inhibitor BIM-1 (10 nM and 1 μM), the PKCα/βII inhibitor GÖ 6076 (2.3 nM), and the PKC δ inhibitor rottlerin (0.5 μM) (Fig. 3A–C). Inhibition of PKCδ showed the most efficient downregulation of vascular areas. By contrast, myr-PKCζ peptide (50 μM) did not influence vascular differentiation in EBs, thus excluding a role of PKCζ in vasculogenesis of ES cells (see Fig. 3A–C). Above all, pharmacological inhibition with PKC inhibitors did not affect cardiac marker protein expression (see Fig. 3C; *n*=3) and the percentage of spontaneously contracting EBs (Fig. 3D; *n*=3), thus excluding a role of PKC for commitment of the cardiac cell lineage and pointing towards the notion that PKC regulates a cardiomyogenesis-independent signaling pathway of vasculogenesis.

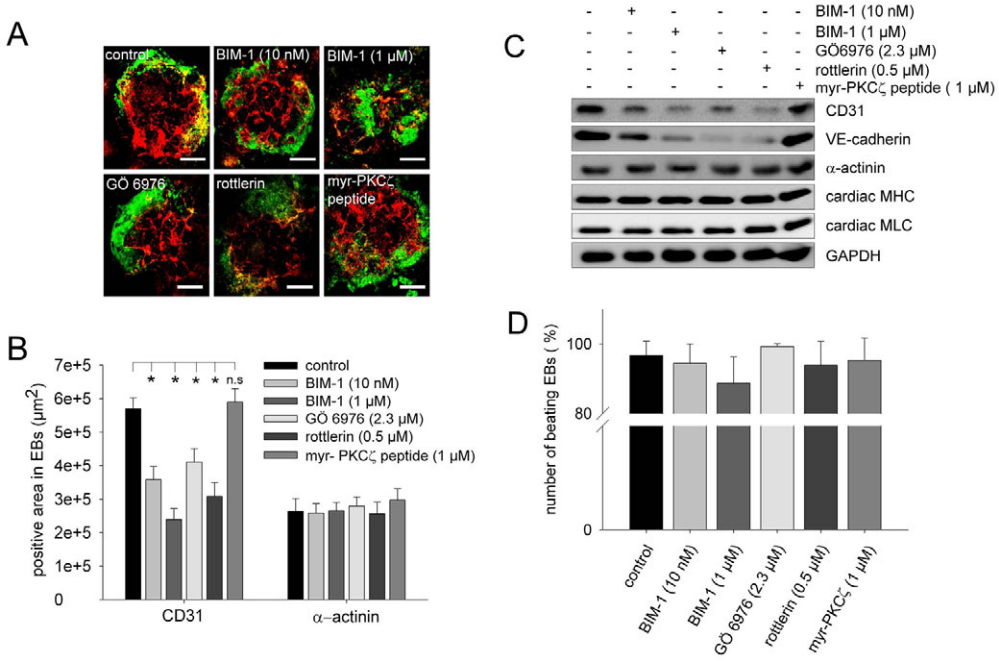

Fig. 3. PKC regulates vasculogenesis but not cardiomyogenesis of ES cells. (**A**,**B**) Treatment of EBs from day 4 to day 10 of cell culture with inhibitors of the PKC family, i.e. BIM-1, GÖ6976 and rottlerin significantly inhibited vascular differentiation (CD31-positive cell areas) (red), whereas myr-PKCζ peptide inhibitor was without effects. By contrast, cardiomyogenesis remained unimpaired following treatment of EBs with BIM-1, GÖ6976, rottlerin, or myr-PKCζ peptide inhibitor as shown by α-actinin immunohistochemistry (green). Scale bars: 100 μm. (**C**) Western blot analysis demonstrated that PKC inhibitors with the exception of myr-PKCζ peptide inhibitor downregulated the vascular cell markers CD31 and VE-Cadherin, whereas expression of the cardiac markers cardiac MHC and MLC was preserved. GAPDH expression was used as internal standard. (**D**) The number of beating EBs was not changed upon treatment with PKC inhibitors. **P*<0.05, as indicated.

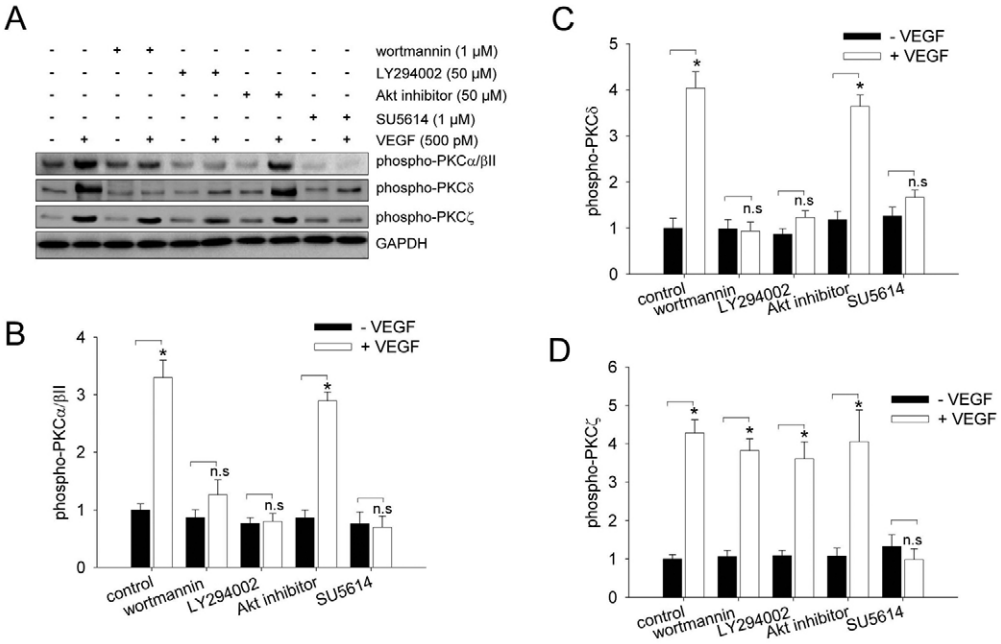

Fig. 4. Activation of PKC isoforms by VEGF is dependent on PI3K but not Akt. (**A**–**D**) PI3K inhibitors wortmannin or LY294002 significantly inhibited the activation of PKCα/βII (A,B) and PKCδ (A,C) but not PKCζ (A,D) in EBs stimulated by VEGF, as evaluated by western blot analysis. Treatment of EBs with SU5614 totally blunted the activation of PKC. Treatment of the EBs with Akt inhibitor did not affect PKC activation. GAPDH expression was used as internal standard. The bar charts represent quantitative analyses of western blot experiments. **P*<0.05, as indicated.

Activation of PKC by VEGF is dependent on the PI3K–PDK1 pathway but independent of Akt

To elucidate a potential crosslink between the VEGF-PI3K pathway and activation of PKC, EBs were incubated during the time of VEGF treatment with wortmannin (1 μM), LY294002 (50 μM), Akt inhibitor (50 μM) and VEGF receptor inhibitor SU5614 (1 μM) at day 5 of cell culture (Fig. 4A–D; *n*=3). It was apparent that PI3K inhibition significantly abolished the VEGF activation of PKCα/βII (Fig. 4A,B) and PKCδ (Fig. 4A,C) but not of PKCζ (Fig. 4A,D). Treatment the EBs with SU5614 totally blunted the activation of PKC (Fig. 4A–D), suggesting a significant role of Flk-1 in activation of all PKC isoforms. By contrast, treatment the EBs with Akt inhibitor did not affect PKC activation by VEGF (see Fig. 4A–D), thus indicating that activation of PKC by VEGF is mediated via PI3K but not by Akt downstream of PI3K. Furthermore our data strongly support a role of PKCα/βII and PKCδ downstream of PI3K for cardiac-independent vasculogenesis of ES cells.

It is well established that Akt and phosphoinositide-dependent kinase-1 (PDK1) are downstream targets of PI3K and both become active through phosphorylation (Cantley, 2002; Gerasimovskaya et al., 2005). To investigate the involvement of Akt and PDK1 in VEGF-mediated regulation of the PI3K–PKC pathway, in a first step the phosphorylation of Akt and PDK1 was investigated using pharmacological inhibition of PI3K and PKC. After VEGF stimulation of 5-day-old EBs, Akt (Fig. 5A) and PDK1 (Fig. 5B) became phosphorylated within 10 minutes, which was blunted after preincubation with the PI3K inhibitors wortmannin or LY294002. By contrast, preincubation with the general PKC inhibitor BIM-1 did not reduce Akt or PDK1 phosphorylation (Fig.

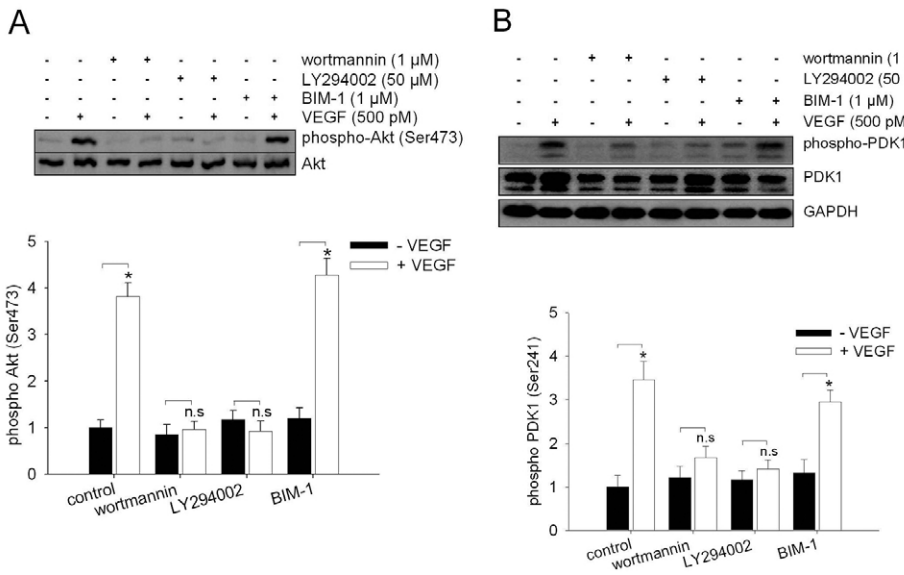

Fig. 5. Stimulation of PDK1 and Akt phosphorylation upon VEGF treatment is dependent on PI3K but not PKC. (**A**,**B**) In response to VEGF stimulation, Akt (A) and PDK1 (B) became phosphorylated after 10 minutes. This was blunted in 5-day-old EBs preincubated for 2 hours with PI3K inhibitor wortmannin or LY294002. Preincubation of 5-day-old EBs with BIM-1, an inhibitor of the PKC family, failed to reduce Akt or PDK1 phosphorylation. GAPDH expression was used as internal standard. The bar charts represent quantitative analyses of western blot experiments. **P*<0.05, as indicated.

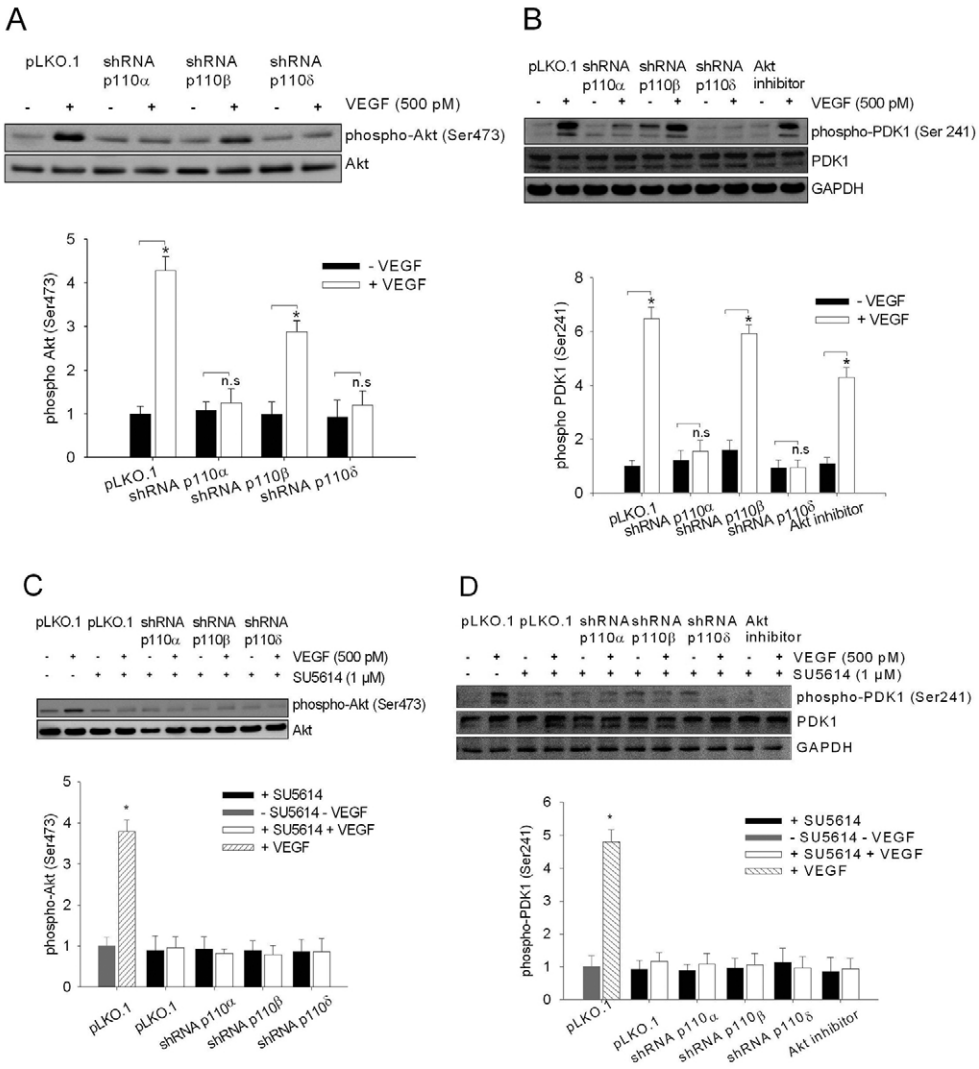

Fig. 6. Akt and PDK1 activation upon VEGF treatment are dependent on VEGFR2–PI3Kα and VEGFR2–PI3Kδ pathways. (**A**,**B**) Activation of PDK1 was not dependent on the Akt pathway. PI3K catalytic subunits p110α and p110δ are essential for Akt and PDK1 phosphorylation in EBs. (**C**,**D**) Treatment of 5-day-old, VEGF-stimulated EBs with SU5614 inhibited Akt and PDK1 phosphorylation. GAPDH expression was used as internal standard. The bar charts represent quantitative analyses of western blot experiments. *P<0.05, as indicated.

5A,B). These findings indicate that stimulation of Akt and PDK1 phosphorylation upon VEGF treatment was mediated through a PI3K pathway but did not depend on PKC.

To further specify the PI3K IA catalytic subunits involved in Akt and PDK1 activation upon treatment of EBs with VEGF, p110α, p110β and p110δ gene-inactivated EBs (5-day-old) were incubated with VEGF and phospho-Akt or phospho-PDK1 and analyzed. VEGF-mediated Akt and PDK1 phosphorylation was absent in p110α and p110δ gene-inactivated cells, whereas stimulation of Akt and PDK1 phosphorylation was still present in p110β gene-inactivated cells (Fig. 6A,B; n=3). In addition, it was found that in the presence of Akt inhibitor VEGF still activated PDK1, thus suggesting that PDK1 activation upon VEGF treatment occurs via a signaling cascade that is independent of Akt and might be located upstream of PKC (Fig. 6B). To further substantiate our observation of a relation between VEGF and the PI3K–Akt–PDK1 signaling pathway, PI3K catalytic subunit gene-inactivated EBs were treated with the Flk-1 inhibitor SU5614 (1 μM) during VEGF treatment. This treatment totally inhibited Akt and PDK1 phosphorylation (Fig. 6C,D; n=3) which clearly shows that Akt and PDK1 are downstream of Flk-1. Taken together, our data demonstrate that Akt and PDK1 act downstream of the Flk-1–PI3K pathway and might regulate independent signaling pathways of cardiac-associated versus cardiac-independent vasculogenesis.

Cardiomyogenesis and cardiac-associated vasculogenesis are regulated by a PI3K–Akt signaling pathway

The data from the present study demonstrate that VEGF stimulation of differentiating ES cells resulted in cardiac and vascular differentiation. We also demonstrated that inhibition of PKC isoforms abolished vasculogenesis, whereas cardiomyogenesis was maintained. Because PKC inhibition did not exert effects on VEGF-mediated Akt phosphorylation, we hypothesized that cardiomyogenesis and cardiomyogenesis-associated vasculogenesis might be regulated by a PI3K–Akt-dependent but PKC-independent pathway. To determine whether Akt was involved in cardiomyogenesis-associated vasculogenesis, EBs were incubated with Akt inhibitor in the presence or absence of VEGF. This treatment resulted in a significant decrease in cardiac as well as vascular differentiation in EBs preincubated with Akt inhibitor, as evidenced by immunohistochemistry (Fig. 7A,B; n=3) and western blot analysis (Fig. 7C; n=3). When the percentage of spontaneously contracting EBs was assessed, it was apparent that inhibition of

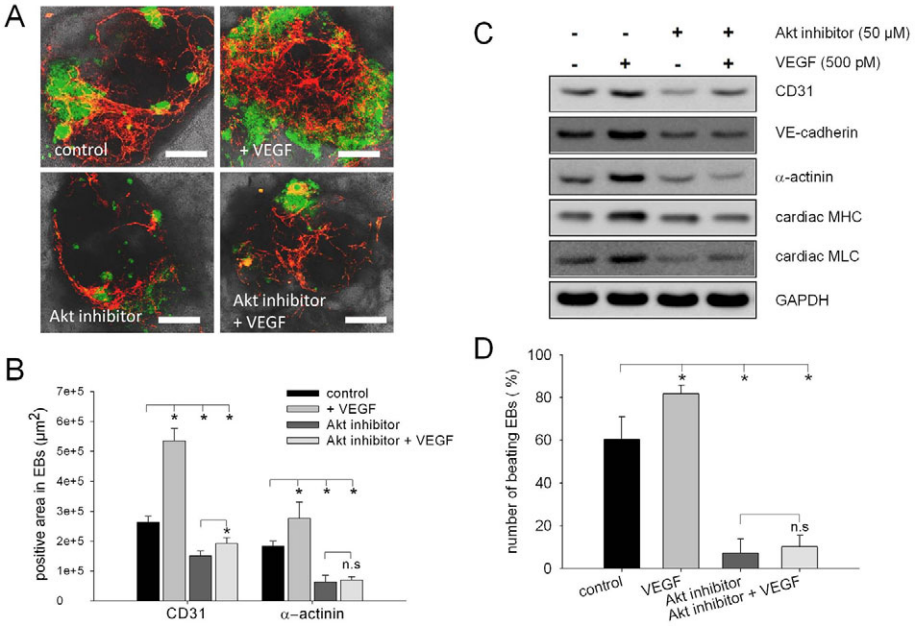

Fig. 7. Akt is required for VEGF-stimulated cardiovascular differentiation of ES cells. (**A**,**B**) Upon incubation with Akt inhibitor, positive areas of CD31 (red) and α-actinin (green) in EBs preincubated with VEGF (500 pM) were significantly decreased. Scale bars: 100 µm. (**C**) Consequently, endothelial (i.e. CD31, VE-Cadherin) and cardiac-specific (i.e. α-actinin, cardiac MLC, MHC) markers were decreased in western blots. GAPDH expression was used as internal standard. (**D**) Analysis of spontaneously beating EBs following treatment with Akt inhibitor. Note that Akt inhibition significantly decreased cardiovascular differentiation, accompanied by significant decrease in the percentage of beating EBs following treatment with Akt inhibitor from day 4 to day 10. **P*<0.05, as indicated.

Akt resulted in significant reduction of beating EBs down to 7±7% and 10±5% in comparison with control EBs and EBs treated with 500 pM VEGF, respectively. Moreover, the reduction in the percentage of beating EBs could not be restored upon VEGF treatment in the EBs preincubated with Akt inhibitor (Fig. 7D; *n*=3). Taken together, our experiments demonstrate that the VEGF–PI3K–Akt signaling pathway is essential for cardiomyogenesis and vasculogenesis, whereas PCK activated via the VEGF–PI3K pathway is only involved in vasculogenesis.

Pharmacological inhibition of PI3Ks affects cardiovascular gene expression and vascular sprouting of Flk-1$^+$ cells derived from EBs

The effects of pharmacological inhibition of PI3K and PKC signaling on cardiovascular differentiation of pluripotent ES cells might result from unspecific effects exerted on different cell types present in EBs and regulating cardiovascular commitment. We therefore isolated Flk-1$^+$ cardiovascular progenitor cells from 4-day-old EBs by magnetic cell sorting (MACS), i.e. during the culture time when Flk-1 protein expression was maximal (supplementary material Fig. S5A; *n*=3). Fluorescence-activated cell sorting (FACS) resulted in a purity of approximately 91% Flk-1$^+$ cells that expressed the vascular marker CD31 and the cardiac marker Nkx2.5 (supplementary material Fig. S5B; *n*=3). Flk-1$^+$ cells were incubated with the PI3K inhibitor wortmannin (1 µM); the class 1A PI3K catalytic subunits inhibitors compound 15e (0.5 µM), TGX-221 (1 µM) and IC-87114 (1 µM); the PKC inhibitors BIM-1 (1 µM), GÖ 6976 (2.3 nM), rottlerin (0.5 µM) and myr-PKCζ peptide (50 µM); and Akt inhibitor (50 µM) in the presence or absence of VEGF (500 pM). Subsequently, mRNA expression of Flk-1, the vascular markers CD31 and VE-Cadherin, the cardiac transcription factors Nkx2.5 and GATA4, and the cardiac genes encoding MLC2v, cTNT, α-MHC and β-MHC were analyzed (Fig. 8A–F; *n*=3). Additionally, EBs grown from Flk$^+$ cells were cultivated from day 4 until day 10 on a matrigel-coated surface, and were exposed to the PI3K and PKC inhibitors (Fig. 9A,B; *n*=3). A clear reduction in vascular sprout formation and reduction in mRNA expression, analyzing typical endothelial as well as early and late cardiac genes, were found after exposure to wortmannin (1 µM), 15e (0.5 µM) and IC-87114 (1 µM) either in the presence or absence of VEGF. In corroboration of our results achieved with unselected ES cell inhibition of PKC using BIM-1, GÖ 6976 and rottlerin as well as Akt inhibitor abolished the effects of VEGF on vascular marker expression, whereas the myr-PKCζ peptide was without effect. By contrast, cardiac transcription factor and cardiac marker expression was only downregulated upon treatment with PI3K inhibitors (with the exception of TGX-221) and Akt inhibitor, whereas PKC inhibitors were without effect. Notably, Flk-1 expression was downregulated by PI3K inhibitors and Akt inhibitor but not by PKC inhibitors in Flk-1$^+$ cells isolated from EBs. These data clearly support the notion that PI3K catalytic subunits p110α and p110δ are central to cardiovascular differentiation of Flk-1$^+$ progenitor cells, and that Akt downstream of PI3K is involved in both cardiomyogenesis and vasculogenesis, whereas PKC is involved only in vasculogenesis.

Discussion

Recent research has shown that VEGF-regulated signaling pathways are not only involved in vasculogenesis but also in cardiomyogenesis by acting on VEGFR2$^+$ cardiovascular progenitor cells in vitro in ES cells and in vivo in the embryonic heart. These progenitor cells are distinct from VEGFR2$^+$ vasculogenic progenitor cells present in hemangioblasts from the yolk sac (Iida et al., 2005; Kattman et al., 2006), thus raising the issue of how cardiomyogenesis and vasculogenesis are controlled during embryogenesis. PI3K signaling pathways are known to be regulated by VEGF (Hamada et al., 2005; Gliki et al., 2002; Gerber et al., 1998), suggesting the possibility that signaling pathways involved in embryonic vasculogenesis are diversifying downstream of PI3K. Hence, the present study was undertaken to evaluate the role of PI3K class IA (consisting of PI3Kα, PI3Kβ and PI3Kδ isoforms), their downstream effector pathways and their interrelation with the PKC family to dissect the mechanisms of cardiomyogenesis versus vasculogenesis in differentiating ES cells.

Treatment of differentiating ES cells cultures with the PI3K inhibitors wortmannin and LY294002 reduced not only the area of

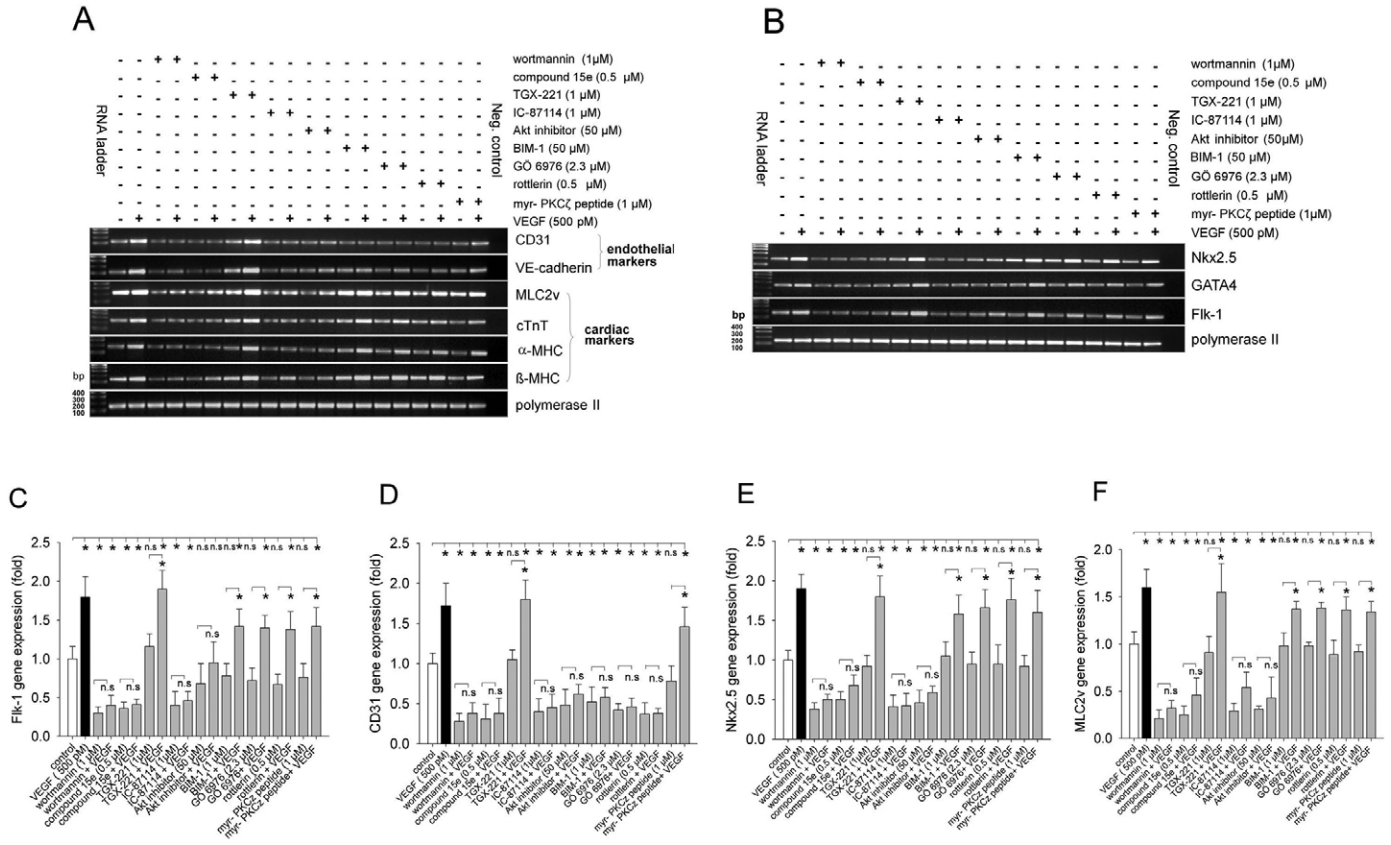

Fig. 8. Gene expression of endothelial and cardiac markers in isolated Flk-1$^+$ cells upon interference with PI3K–PKC signaling. Gene expression was measured in the presence or absence of wortmannin, compound 15e, TGX-221, IC-87114, Akt inhibitor, BIM-1, GÖ 6976, rottlerin and myr-PKCζ peptide. (**A**) mRNA expression analysis in the presence and absence of VEGF of the vascular markers CD31 and VE-Cadherin as well as the cardiac genes MLC2v, cTNT, α-MHC and β-MHC. (**B**) mRNA expression analysis in the presence and absence of VEGF of Flk-1 and the cardiogenic transcription factors Nkx2.5 and GATA-4. Cells were treated with substances from day 4 to day 10 of cell culture. Polymerase 2 was used as internal standard. (**C**–**F**) Semiquantitative analysis of Flk-1 (see C), CD31 (see D), Nkx2.5 (see E), and MLC2v (see F) mRNA expression as evaluated from RT-PCR plots. *P<0.05, as indicated.

α-actinin-positive cardiomyocytes and the number of EBs containing beating foci, but also cell areas of blood vessel-like structures. These results were consistent with the previously demonstrated link between PI3K and cardiovascular differentiation during embryogenesis and ES cell differentiation (Shiojima and Walsh, 2002; Klinz et al., 1999; Jiang et al., 2000; Sauer et al., 2000).

PI3Ks probably stimulate cardiovascular differentiation through either one or several isoforms. In this context, we demonstrated that targeting class IA PI3K catalytic subunit p110 using specific pharmacological inhibitors for p110α (compound 15e) and p110δ (IC-87114) impaired cardiovascular differentiation, whereas inhibition of p110β by TGX-221 was without significant effects. We additionally applied the shRNA technique to knockdown class IA PI3K isoforms in ES cells. Unexpectedly, it was observed that silencing the p110β subunit led to upregulation of p110α, which might reflect a potential cross-talk of the p110β and p110α isoforms. It has been previously reported that, in some cases, deletion of the PI3K regulatory subunits also impaired the expression level of the corresponding catalytic subunits. Similarly, it has been shown that knockout of genes encoding the class IA p110 catalytic subunits leads to an overexpression of the regulatory subunits, which might block or activate other signaling pathways (Vanhaesebroeck et al., 1997).

Previous studies showed that VEGF is essential for cardiomyogenesis and/or vascular differentiation (Chen et al., 2006; Cheung, 1997; Gerber et al., 1998; Gliki et al., 2002; Song et al., 2007; Yang et al., 2002; Zisa et al., 2009; Lange et al., 2009; Bekhite et al., 2010) and might activate PI3K (Bos 1995; Gerber et al., 1998). Moreover, it has been suggested that Flk-1$^+$ plays a crucial role in regulating PI3K activity in endothelial cells (Gerber et al., 1998; Ferrara, 1999; Thomas and Owen, 2008). To investigate whether the PI3K class IA isoforms are essential for VEGF–Flk-1$^+$ signaling pathways leading to cardiovascular differentiation, PI3K p110 isoform gene-inactivated ES cells were treated with VEGF. A significant increase in cardiovascular differentiation upon treatment of EBs with VEGF was observed in the pLKO.1 EBs (negative control) and P110β knockdown cell lines. By contrast, the increase of cardiomyogenesis and vascular differentiation by VEGF was totally blunted after silencing the p110α and p110δ subunits, thus corroborating our experiments with specific pharmacological inhibitors of p110α and p110δ. These data were consistent with previously published data demonstrating that PI3K determines heart size in mice (Shioi et al., 2000). Furthermore, the data corroborated studies of others demonstrating that PI3K class IA was required for cardiovascular differentiation in mouse embryos in the presence of VEGF (Luo et al., 2005; Yuan et al., 2008), and in differentiating ES cells where VEGF was found to significantly

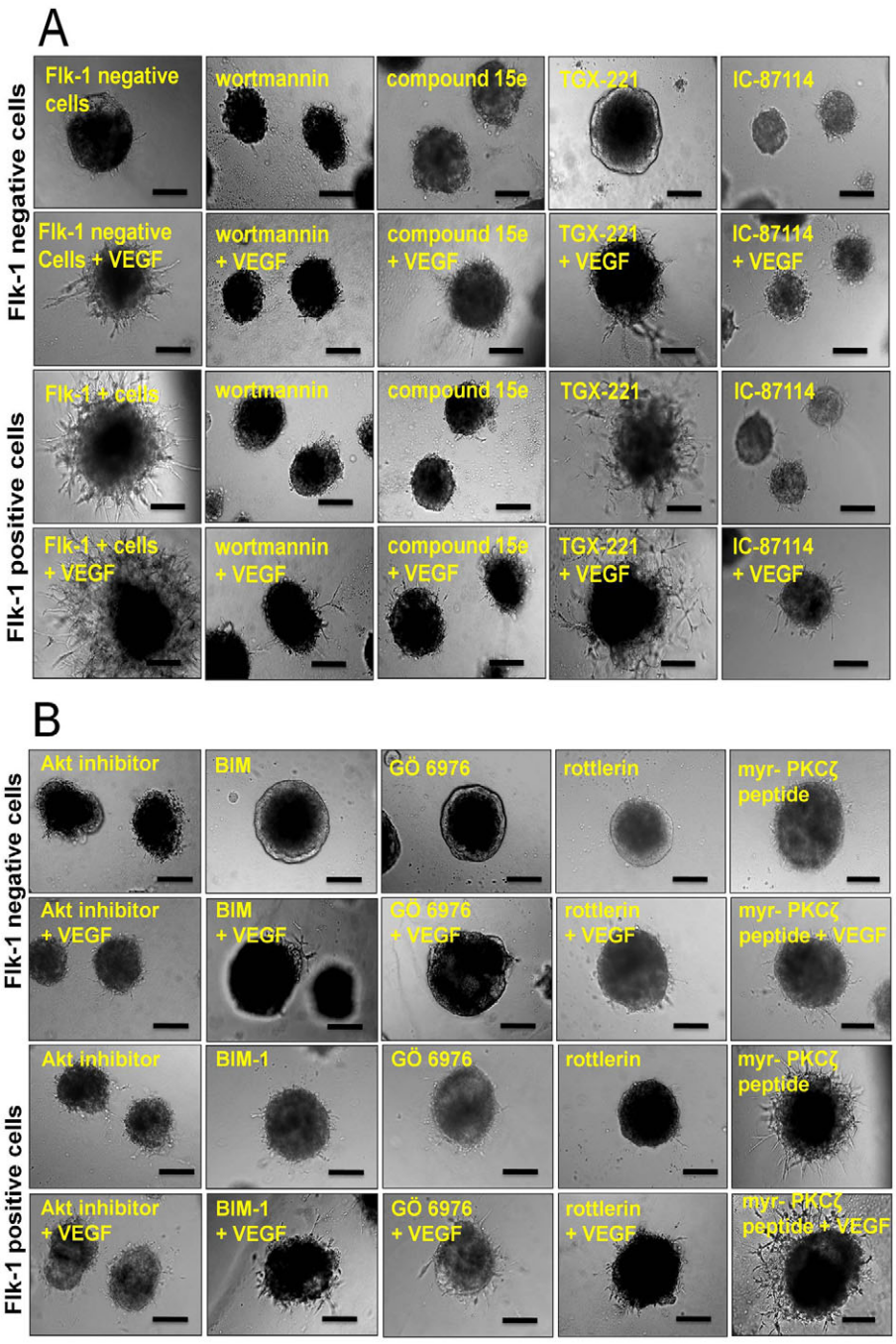

Fig. 9. Vascular sprouting from Flk-1$^+$ EBs upon interference with PI3K–PKC signaling. EBs were cultivated from Flk-1 negative and positive cells and grown on matrigel-coated surface. Vascular outgrowth was analyzed upon incubation with inhibitors either in the presence or absence of VEGF (500 pM) from day 4 to day 10 of cell culture. (**A**) Representative transmission images of EBs on matrigel upon treatment with the PI3K inhibitors wortmannin (1 μM), compound 15e (0.5 μM), TGX-221(1 μM), and IC-87114 (1 μM). (**B**) Representative transmission images of EBs on matrigel upon treatment with Akt inhibitor (50 μM) as well as the PKC inhibitors BIM-1 (50 μM), GÖ 6976 (2.3 μM), rottlerin (0.5 μM), and myr-PKCζ peptide (1 μM). Note that vascular sprouting was efficiently inhibited in the presence of the nonspecific PI3K inhibitor wortmannin, the p110α inhibitor compound 15e, and the p110δ inhibitor IC-87114. Vascular sprouting was inhibited in the presence of Akt and PKC inhibition with the exception of myr-PKCζ peptide, which inhibits PKCζ.

enhance α-MHC, cardiac troponin I and Nkx2.5 expression (Chen et al., 2006).

To investigate the interrelation between the PI3K signaling pathway and PKC activation and its impact for cardiomyogenesis versus vasculogenesis, ES cells were treated with inhibitors of the PKC family. It was evident that BIM-1, GÖ 6976 and rottlerin significantly inhibited vasculogenesis. Notably, cardiomyogenesis remained unimpaired after treatment of EBs with BIM-1, GÖ 6976, rottlerin or myr-PKCζ peptide inhibitor, which indicates that the signaling pathways regulating cardiac and vascular differentiation of ES cells diversify downstream of PI3K. It furthermore demonstrated that PKC downstream of PI3K regulates vasculogenesis but is not involved in cardiomyogenesis. The importance of PKC regarding the VEGF-mediated PI3K pathway was previously suggested by several authors (Chou et al., 1998; Dutil et al., 1998; Gliki et al., 2002; Le Good et al., 1998). Others provided direct evidence that the PI3K–Akt pathway is required for VEGF to induce endothelial cells, but a possible involvement of PKC was not specified (Shiojima and Walsh, 2002; Abid et al., 2004; Qi and Claesson-Welsh, 2001).

To elucidate a potential crosslink between the VEGF–PI3K pathway and activation of PKC, EBs were incubated during the time of VEGF treatment with general PI3K inhibitors, which significantly blunted the activation of PKCα/βII and PKCδ but not of PKCζ. Treatment of EBs with SU5614 totally abolished the activation of all PKC isoforms, thus confirming that VEGF-

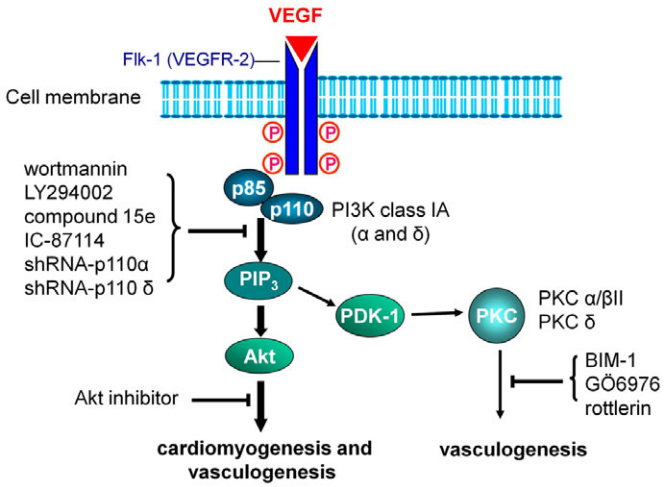

Fig. 10. Signaling pathway of VEGF-mediated cardiomyogenesis and vasculogenesis in mouse ES cells. VEGF binding to Flk-1 (VEGFR2) activates PI3K class IA (p110α and p100δ catalytic subunits). Downstream of PI3K, Akt and PDK1 are independently activated. Akt downstream of PI3K regulates vasculogenesis as well as cardiomyogenesis, whereas PDK-1–PCK (PKC α/βII, PKC δ) regulates only vasculogenesis without playing a role in cardiomyogenesis.

mediated PKC activation occurs via Flk-1. By contrast, treatment of EBs with Akt inhibitor did not affect PKC activation by VEGF, which indicates that the PI3K–Akt pathway is apparently distinct from the PI3K–PKC axis. Furthermore, PDK1 was activated in response to VEGF treatment independently of Akt, as indicated by the fact that PDK1 phosphorylation upon VEGF treatment still occurred in the presence of Akt inhibitor. This finding suggests that the VEGF–PI3K–PDK1 signaling cascade is acting upstream of PKCα/βII and PKCδ in differentiating EBs. Comparable data have been previously obtained in human embryonic kidney cells, where PKC isotypes were controlled by PI3K through PDK1 (Le Good et al., 1998).

Because inhibition of PKC abolished vasculogenesis but not cardiomyogenesis of ES cells we hypothesized that cardiomyogenesis-associated vasculogenesis might utilize a PKC-independent signaling pathway, which could be regulated by Akt downstream of PI3K. Akt-dependent signaling pathways are known to be involved in the regulation of cardiac growth, contractile function, and coronary angiogenesis (Shiojima and Walsh, 2006; Fujio et al., 2000; Matsui et al., 1999), suggesting a possible role in pathways regulating cardiovascular differentiation from cardiovascular progenitor cells in the heart. Our assumption was firstly supported by experiments demonstrating that silencing of the PI3K catalytic subunits p110α and p110δ abolished VEGF-mediated Akt and PDK1 phosphorylation and likewise blunted PKC activation, which is presumably mediated via the PDK1 pathway. Secondly, we observed that treatment of VEGF-stimulated EBs cells with Akt inhibitor not only inhibited vasculogenesis but also cardiomyogenesis, despite a persistent PKC activation under these experimental conditions. Hence, our findings show that Akt regulates cardiovascular differentiation downstream of the PI3K in VEGF-stimulated ES cells.

The biological activities of VEGF are mainly mediated by Flk-1 (Ferrara et al., 2003), which is one of the early lateral mesoderm markers, when cardiomyogenesis occurs. It has been shown that Flk-1$^+$ cells could act as cardio-hemangioblasts to form cardiomyocytes as well as endothelial cells as constituents of the coronary and endocardial vasculature (Iida et al., 2005; Kattman et al., 2006). Our results demonstrate that VEGF via Flk-1 stimulates the PI3K–Akt axis to initiate cardiomyogenesis as well as cardiac-associated vasculogenesis, and stimulates PI3K–PDK1–PKC signaling to promote vasculogenesis independently of cardiomyogenesis. These effects are not only present in whole mount EBs, which differentiate various potentially interacting cell types of all three germ layers, but also in isolated Flk-1$^+$ cardiovascular progenitor cells. More precisely, the data from our study demonstrate that PI3K class IA isoforms PI3Kα and PI3Kδ control cardiomyogenesis and vascular differentiation, whereas PKCα/βII and PKCδ are involved in vasculogenesis upon VEGF treatment but are dispensable for cardiomyogenesis (see Fig. 10). Apparently, signaling pathways in cardiomyogenesis and vasculogenesis are closely associated to synchronize and phase-lock the development of the cardiovascular system.

Materials and Methods

ES cell culture

The ES cell line CGR8 was obtained from the European Collection of Cell Cultures (ECACC) Wiltshire, UK and cultured on gelatine-coated cell culture flasks in Glasgow minimal essential medium (GMEM; Sigma-Aldrich, Taufkirchen, Germany). Medium was supplemented with 10% inactivated fetal bovine serum (FBS) (Sigma-Aldrich), 2 mM L-glutamine (Biochrom, Berlin, Germany), 45 μM 2-mercaptoethanol (Sigma-Aldrich) and 103 U/ml leukaemia inhibitory factor (LIF) (Chemicon, Hampshire, UK) in a humidified environment containing 5% CO_2 at 37°C. At day 0 of differentiation, adherent cells were enzymatically dissociated using 0.25% trypsin with 1 mM ethylenediaminetetraacetic acid (EDTA) in Hanks' balanced salt solution (HBSS) (Invitrogen, Karlsruhe, Germany). A total of 1×10^7 cells was seeded into spinner flasks (Integra Biosciences, Fernwald, Germany) containing 125 ml Iscove's medium supplemented with the same additives as described above but devoid of LIF. Following 24 hours, 125 ml medium was added to give a final volume of 250 ml. The spinner flask medium was stirred at 20 r.p.m. using a stirrer system (Integra Biosciences), and 125 ml cell culture medium was exchanged every day.

The growing EBs were removed from spinner flasks and plated at day four of culture either on tissue culture dishes (Greiner Bio-One, Frickenhausen, Germany) or for enhanced cardiac differentiation on 'lumox' cell culture dishes (Sigma-Aldrich).

Downregulation of PI3K p110 catalytic subunit using shRNA technique

pLKO.1-puro (Sigma-Aldrich) derivative plasmid carrying shRNA sequence targeting PI3K p110α catalytic subunit, shRNA targeting PI3K p110β catalytic subunit, and shRNA targeting PI3K p110δ catalytic subunit were separately introduced into CGR8 cells by lentiviral particles. Transduced cells with pLKO.1 vector (containing non-hairpin insert) were used as negative control. Lentiviral particles were generated using human embryonic kidney (HEK) 293FT-based amphotropic Phoenix packaging cells (Phoenix-Ampho, Invitrogen, Karlsruhe, Germany) cultured in DMEM, 10% fetal calf serum, 1% sodium pyruvate plus penicillin/streptomycin. For a 10-cm dish, lentiviral vector plasmids (10 μg) were co-transfected with plasmids encoding the HIV-Rev (5 μg), HIV-MDL (10 μg) and the ecotropic envelope (2 μg) in the presence of polyethylenimine (70 μg, Sigma-Aldrich). Supernatants were harvested after 24 hours and 48 hours and filtered through 0.22-mm filters (Schambach et al., 2006). Supernatants were added to CGR8 cells, which were plated in a well of a 6-well plate, centrifuged at 400 ***g*** in a cell culture centrifuge for 1 hour and replaced with fresh medium on the following day. A total of three infection rounds were carried out within 48 hours. On the following day, the cells were passaged and selected with 2 μg/ml puromycin (Sigma-Aldrich) for 10–14 days. Transduction effectiveness was assessed via GFP control vector (Sigma-Aldrich) as previous described (Sauer et al., 2008). Downregulation of PI3K p110 catalytic subunit p110α, p110β and p110δ was analyzed by RT-PCR and western blot.

Treatment with inhibitors

EBs differentiated from ES cells were incubated in the presence or absence of VEGF-165 (500 pM) (Sigma-Aldrich) with wortmannin (1 μM), LY294002 (50 μM) (both from Cell Signaling Technology, Frankfurt, Germany), bisindolylmaleimide I (BIM-1) (10 nM/1 μM) (Calbiochem, Bad Soden, Germany), or with inhibitors of p110 catalytic subunit by using compound 15e (0.5 μM), TGX-221 (1 μM) (Alexis Biochemicals, Lörrach, Germany) and IC87114 (1 μM), which have been shown to inhibit PI3K p110α, PI3K p110β and PI3K p110δ, respectively (Hayakawa et al., 2006; Jackson et al., 2005; Lee et al., 2006). PKC was inhibited by Gö6976 (2.3

nM), which is specific for PKCα/βII (Sigma-Aldrich), myristoylated PKCζ peptide (myr-PKCζ peptide) inhibitor (50 μM) (Biomol, Lörrach, Germany), and rottlerin (0.5 μM), which is a specific inhibitor of PKCδ (Biomol). Furthermore, Akt inhibitor (50 μM) (Calbiochem) and SU5614 (1 μM) inhibitor for VEGF receptor (Calbiochem) were used in the study.

Reverse transcription-polymerase chain reaction

Total RNA isolation from ES cells was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's recommended procedures, followed by genomic DNA digestion using DNaseI (Invitrogen, Karlsruhe, Germany). cDNA was synthesized from 2 μg total RNA using a superscript II reverse transcriptase (Invitrogen) according to manufacturer's instructions and using random primer. The reverse transcription product was diluted 1:10, and PCR was performed with following primers (Sigma, Geonysis, Germany): CD31: sense 5′-GAGCCCAATCACGTTTCAGTT-3′, antisense 5′-TCCTTCCTGCTTCTTGCTAGCT-3′; VE-Cadherin: sense 5′-GTCAGCTATAGGGACCTCTGT-3′, antisense 5′-TCATTTCCTTTCACGATTTGG-3′; Nkx2.5: sense 5′-CCACTCTCGCTACCCACCT-3′, antisense 5′-CCAGGTTCAGGATGTCTTTGA-3′; Flk-1: sense 5′-GTGGACCAAATGCCTGACTC-3′, antisense 5′-TTCTGTTCTGTTGGCCCTTT-3′; α-MHC: sense 5′-TGAAAACGGAAAGACGGTGA-3′, antisense 5′-TCCTTGAGGTTGTACAGCACA-3′; β-MHC: sense 5′-CTACAGGCCTGGGCTTACCT-3′, antisense 5′-TCTCCTTCTCAGACTTCCGC-3′; MLC2v: sense 5′-AAAGAGGCTCCAGGTCCAAT-3′, antisense 5′-CCTCTCTGCTTGTGTGGTCA-3′; GATA4: sense 5′-ACTCTGGAGGCGAGATGGG-3′, antisense 5′-GACACCGCAGCATTACGGCTC-3′; PI3k p110α: sense 5′-ACTGTTCAGAGAGGCCAGGA-3′, antisense 5′-CGGTTGCCTACTGGTTCAAT-3′; PI3k p110β: sense 5′-AGCTGGTCTTCGTTTCCTGA-3′, antisense 5′-TCCACCACGACTTGACACAT-3′; PI3k p110δ: sense 5′-CTGACCCCTCATCTGACCAT-3′, antisense 5′-TCGTCAGCATTCACTTTTCG-3′; polymerase II: sense 5′-GACAAAACTGGCTCCTCTGC-3′, antisense 5′-GCTTGCCCTCTACATTCTGC-3′; Nestin: sense 5′-CGGCCCACGCATCCCCCATCC-3′, antisense 5′-AGCGGCCTTCCAATCTCTGTTCC-3′; Neuron-specific enolase: sense 5′-CCAAGTCACCCAGAACACCT-3′, antisense 5′-AAACACCCCAACACACCAAT-3′.

The PCR product was amplified using 40 cycles performed at 58°C annealing temperature. Gel images were subsequently captured using a 1% agarose gel.

Western blot assay

The western blot assays were carried out after washing the ES cells in cold PBS and lysing in lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and 1 mM Na_3VO_4) that contained 1 mM phenylmethylsulfonyl fluoride for 30 minutes on ice. Samples were centrifuged at 13.000 ***g*** for 10 minutes to pellet the debris. After determination of protein concentrations using the method of Bradford, 40 μg of protein per sample was heated to 95°C for 10 minutes and separated in SDS polyacrylamide gels and transferred to nitrocellulose membranes at 20 V over 10 hours. Membranes were blocked with 20% (wt/vol) dry fat-free milk powder in Tris-buffered saline with 0.1% Tween (TBST) for 5 hours at 4°C. After that, primary antibodies were incubated for 10 hours at 4°C. Subsequently membranes were washed with 0.1% TBST. For the secondary antibody reaction, membranes were incubated with the adequate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) for 60 minutes at room temperature. Corresponding bands were visualized using ECL detection kit (Amersham, Freiburg, Germany) and captured by a digital imaging system (LAS 3000, Fujifilm, Japan). The following primary antibodies were used for western blot analyses: polyclonal goat-anti mouse phospho-PKCδ (Ser643) and polyclonal rabbit anti mouse phospho-PKCζ (Thr410), PI3K p110α, PI3K p110β and PI3K p110δ were all obtained from Santa Cruz Biotechnology. The monoclonal rabbit anti-mouse antibodies directed against phospho-PKCα/β (Thr638/641), phospho-PDK1 (Ser241) and phospho-Akt (Ser473) as well as the polyclonal rabbit anti mouse PDK1 and Akt antibodies were all obtained from Cell Signaling Technologies (Frankfurt, Germany). For cardiomyocyte differentiation, a monoclonal mouse anti-mouse sarcomeric α-actinin (Sigma-Aldrich), a monoclonal mouse anti-mouse myosin heavy chain (MHC), and a monoclonal anti-myosin light chain (MLC) antibody were used (both from Abcam, Cambridge, UK). For assessing endothelial differentiation, a monoclonal rabbit anti mouse Flk-1 antibody from Cell Signaling Technologies, a monoclonal mouse anti-mouse CD31 antibody and a polyclonal rabbit anti-mouse VE-Cadherin antibody (both from Abcam) were used.

Immunohistochemistry and quantitative analysis

For immunofluorescence stainings, the ES cells were fixed in methanol–acetone (7:3) for 1 hour at –20°C. Rat monoclonal anti-CD31 (Chemicon, Schwalbach, Germany) and monoclonal mouse anti-sarcomeric α-actinin (Sigma-Aldrich) were used. Fluorescence recordings were performed using a confocal laser scanning microscope (LSM 510; Carl Zeiss, Germany) connected to an inverted microscope (Axiovert 135; Carl Zeiss). Quantitative IHC was analyzed as previously described (Sauer et al., 2005) using an Axiovision software tool (Carl Zeiss). Briefly, images (512×512 pixels) were acquired either from CD31-stained or α-actinin-stained EBs corrected for background fluorescence using the extended depth of focus algorithm of the confocal setup. Generally, four full-frame images separated by a distance of 8 μm in the *z*-direction were recorded that included information on the capillary or cardiac area and spatial organization in a tissue slice 32 μm thick. From the acquired images, an overlay image giving a three-dimensional projection of the vascular and cardiac structures in the scanned tissue slice was generated. By use of the image analysis facilities of the confocal setup, the antigen-positive vascular and cardiac cell areas within the three-dimensional projection of vascular and cardiac structures were identified and related to the cross-section of the respective EB.

MACS and FACS analysis

CGR8 EBs were generated and cultivated as described above. The 4-day-old EBs were dissociated by incubation with IK-buffer containing collagenase type II (4 mg/ml, PAA, Coelbe, Germany) at 37°C for 5–10 minutes. For cell separation, MACS columns (Miltenyi Biotec, Bergisch-Gladbach, Germany) were used. The separation procedure was carried out using a PE-conjugated rat anti-mouse Flk-1 antibody (dilution 1:10; BD, Franklin Lakes, NJ). For labeling, anti-PE MicroBeads (all from Miltenyi Biotec) were used. Positive selected cells were placed on a matrigel surface and further cultured. Purity analysis was carried out using a FacsCalibur (Becton Dickinson, Heidelberg, Germany) with a scanning wave length of 488 nm (PE-labeled cells) and a detection wave length of 575 nm.

Statistical analysis

Data in the figures are given as mean values + s.d., with *n* denoting the number of experiments unless otherwise indicated. In each experiment at least 25 EBs were analyzed. GraphPad InStat-3 software (GraphPad Software, San Diego, CA) was applied for One-way ANOVA or Student's *t*-test for unpaired data as appropriate. A value of $P<0.05$ was considered statistically significant. Data are presented as percentage of expression relative to control values, which were set to 100%.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/124/11/1819/DC1

References

Abid, M. R., Guo, S., Minami, T., Spokes, K. C., Ueki, K., Skurk, C., Walsh, K. and Aird, W. C. (2004). Vascular endothelial growth factor activates PI3K/Akt/forkhead signaling in endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **24**, 294-300.

Bekhite, M. M., Finkensieper, A., Abou-Zaid, F. A., El Shourbagy, I. K., Omar, K. M., Figulla, H. R., Sauer, H. and Wartenberg, M. (2010). Static electromagnetic fields induce vasculogenesis and chondro-osteogenesis of mouse embryonic stem cells by reactive oxygen species-mediated upregulation of vascular endothelial growth factor. *Stem Cells Dev.* **19**, 731-743.

Bos, J. L. (1995). A target for phosphoinositide 3-kinase: Akt/PKB. *Trends Biochem. Sci.* **20**, 441-442.

Bu, L., Jiang, X., Martin-Puig, S., Caron, L., Zhu, S., Shao, Y., Roberts, D. J., Huang, P. L., Domian, I. J. and Chien, K. R. (2009). Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. *Nature* **460**, 113-117.

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science* **296**, 1655-1657.

Chen, Y., Amende, I., Hampton, T. G., Yang, Y., Ke, Q., Min, J. Y., Xiao, Y. F. and Morgan, J. P. (2006). Vascular endothelial growth factor promotes cardiomyocyte differentiation of embryonic stem cells. *Am. J. Physiol. Heart Circ. Physiol.* **291**, H1653-H1658.

Cheung, C. Y. (1997). Vascular endothelial growth factor: possible role in fetal development and placental function. *J. Soc. Gynecol. Investig.* **4**, 169-177.

Chou, M. M., Hou, W., Johnson, J., Graham, L. K., Lee, M. H., Chen, C. S., Newton, A. C., Schaffhausen, B. S. and Toker, A. (1998). Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. *Curr. Biol.* **8**, 1069-1077.

Cross, M. J., Dixelius, J., Matsumoto, T. and Claesson-Welsh, L. (2003). VEGF-receptor signal transduction. *Trends Biochem. Sci.* **28**, 488-494.

Dutil, E. M., Toker, A. and Newton, A. C. (1998). Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). *Curr. Biol.* **8**, 1366-1375.

Ferrara, N. (1999). Molecular and biological properties of vascular endothelial growth factor. *J. Mol. Med.* **77**, 527-543.

Ferrara, N., Gerber, H. P. and LeCouter, J. (2003). The biology of VEGF and its receptors. *Nat. Med.* **9**, 669-676.

Fujio, Y., Nguyen, T., Wencker, D., Kitsis, R. N. and Walsh, K. (2000). Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* **101**, 660-667.

Garry, D. J. and Olson, E. N. (2006). A common progenitor at the heart of development. *Cell* **127**, 1101-1104.

Gerasimovskaya, E. V., Tucker, D. A. and Stenmark, K. R. (2005). Activation of phosphatidylinositol 3-kinase, Akt, and mammalian target of rapamycin is necessary for hypoxia-induced pulmonary artery adventitial fibroblast proliferation. *J. Appl. Physiol.* **98**, 722-731.

Gerber, H. P., McMurtrey, A., Kowalski, J., Yan, M., Keyt, B. A., Dixit, V. and Ferrara, N. (1998). Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J. Biol. Chem.* **273**, 30336-30343.

Gliki, G., Wheeler-Jones, C. and Zachary, I. (2002). Vascular endothelial growth factor induces protein kinase C (PKC)-dependent Akt/PKB activation and phosphatidylinositol 3′-kinase-mediates PKC delta phosphorylation: role of PKC in angiogenesis. *Cell Biol. Int.* **26**, 751-759.

Hamada, K., Sasaki, T., Koni, P. A., Natsui, M., Kishimoto, H., Sasaki, J., Yajima, N., Horie, Y., Hasegawa, G., Naito, M. et al. (2005). The PTEN/PI3K pathway governs normal vascular development and tumor angiogenesis. *Genes Dev.* **19**, 2054-2065.

Hayakawa, M., Kaizawa, H., Moritomo, H., Koizumi, T., Ohishi, T., Okada, M., Ohta, M., Tsukamoto, S., Parker, P., Workman, P. et al. (2006). Synthesis and biological evaluation of 4-morpholino-2-phenylquinazolines and related derivatives as novel PI3 kinase p110alpha inhibitors. *Bioorg. Med. Chem.* **14**, 6847-6858.

Iida, M., Heike, T., Yoshimoto, M., Baba, S., Doi, H. and Nakahata, T. (2005). Identification of cardiac stem cells with FLK1, CD31, and VE-cadherin expression during embryonic stem cell differentiation. *FASEB J.* **19**, 371-378.

Jackson, S. P., Schoenwaelder, S. M., Goncalves, I., Nesbitt, W. S., Yap, C. L., Wright, C. E., Kenche, V., Anderson, K. E., Dopheide, S. M., Yuan, Y. et al. (2005). PI 3-kinase p110beta: a new target for antithrombotic therapy. *Nat. Med.* **11**, 507-514.

Jiang, B. H., Zheng, J. Z., Aoki, M. and Vogt, P. K. (2000). Phosphatidylinositol 3-kinase signaling mediates angiogenesis and expression of vascular endothelial growth factor in endothelial cells. *Proc. Natl. Acad. Sci. USA* **97**, 1749-1753.

Katso, R., Okkenhaug, K., Ahmadi, K., White, S., Timms, J. and Waterfield, M. D. (2001). Cellular function of phosphoinositide 3-kinases: implications for development, homeostasis, and cancer. *Annu. Rev. Cell Dev. Biol.* **17**, 615-675.

Kattman, S. J., Huber, T. L. and Keller, G. M. (2006). Multipotent flk-1+ cardiovascular progenitor cells give rise to the cardiomyocyte, endothelial, and vascular smooth muscle lineages. *Dev. Cell* **11**, 723-732.

Kattman, S. J., Adler, E. D. and Keller, G. M. (2007). Specification of multipotential cardiovascular progenitor cells during embryonic stem cell differentiation and embryonic development. *Trends Cardiovasc. Med.* **17**, 240-246.

Klinz, F., Bloch, W., Addicks, K. and Hescheler, J. (1999). Inhibition of phosphatidylinositol-3-kinase blocks development of functional embryonic cardiomyocytes. *Exp. Cell Res.* **247**, 79-83.

Lange, S., Heger, J., Euler, G., Wartenberg, M., Piper, H. M. and Sauer, H. (2009). Platelet-derived growth factor BB stimulates vasculogenesis of embryonic stem cell-derived endothelial cells by calcium-mediated generation of reactive oxygen species. *Cardiovasc. Res.* **81**, 159-168.

Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P. and Parker, P. J. (1998). Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science* **281**, 2042-2045.

Lee, K. S., Lee, H. K., Hayflick, J. S., Lee, Y. C. and Puri, K. D. (2006). Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *FASEB J.* **20**, 455-465.

Luo, J., McMullen, J. R., Sobkiw, C. L., Zhang, L., Dorfman, A. L., Sherwood, M. C., Logsdon, M. N., Horner, J. W., DePinho, R. A., Izumo, S. et al. (2005). Class IA phosphoinositide 3-kinase regulates heart size and physiological cardiac hypertrophy. *Mol. Cell. Biol.* **25**, 9491-9502.

Matsui, T., Li, L., del Monte, F., Fukui, Y., Franke, T. F., Hajjar, R. J. and Rosenzweig, A. (1999). Adenoviral gene transfer of activated phosphatidylinositol 3′-kinase and Akt inhibits apoptosis of hypoxic cardiomyocytes in vitro. *Circulation* **100**, 2373-2379.

Qi, J. H. and Claesson-Welsh, L. (2001). VEGF-induced activation of phosphoinositide 3-kinase is dependent on focal adhesion kinase. *Exp. Cell Res.* **263**, 173-182.

Sauer, H., Rahimi, G., Hescheler, J. and Wartenberg, M. (2000). Role of reactive oxygen species and phosphatidylinositol 3-kinase in cardiomyocyte differentiation of embryonic stem cells. *FEBS Lett.* **476**, 218-223.

Sauer, H., Bekhite, M. M., Hescheler, J. and Wartenberg, M. (2005). Redox control of angiogenic factors and CD31-positive vessel-like structures in mouse embryonic stem cells after direct current electrical field stimulation. *Exp. Cell Res.* **304**, 380-390.

Sauer, H., Ruhe, C., Muller, J. P., Schmelter, M., D'Souza, R. and Wartenberg, M. (2008). Reactive oxygen species and upregulation of NADPH oxidases in mechanotransduction of embryonic stem cells. *Methods Mol. Biol.* **477**, 397-418.

Schambach, A., Galla, M., Modlich, U., Will, E., Chandra, S., Reeves, L., Colbert, M., Williams, D. A., von Kalle, C. and Baum, C. (2006). Lentiviral vectors pseudotyped with murine ecotropic envelope: increased biosafety and convenience in preclinical research. *Exp. Hematol.* **34**, 588-592.

Shioi, T., Kang, P. M., Douglas, P. S., Hampe, J., Yballe, C. M., Lawitts, J., Cantley, L. C. and Izumo, S. (2000). The conserved phosphoinositide 3-kinase pathway determines heart size in mice. *EMBO J.* **19**, 2537-2548.

Shiojima, I. and Walsh, K. (2002). Role of Akt signaling in vascular homeostasis and angiogenesis. *Circ. Res.* **90**, 1243-1250.

Shiojima, I. and Walsh, K. (2006). Regulation of cardiac growth and coronary angiogenesis by the Akt/PKB signaling pathway. *Genes Dev.* **20**, 3347-3365.

Song, Y. H., Gehmert, S., Sadat, S., Pinkernell, K., Bai, X., Matthias, N. and Alt, E. (2007). VEGF is critical for spontaneous differentiation of stem cells into cardiomyocytes. *Biochem. Biophys. Res. Commun.* **354**, 999-1003.

Stephens, L. R., Hughes, K. T. and Irvine, R. F. (1991). Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils. *Nature* **351**, 33-39.

Teicher, B. A. (2006). Protein kinase C as a therapeutic target. *Clin. Cancer Res* **12**, 5336-5345.

Thakker, G. D., Hajjar, D. P., Muller, W. A. and Rosengart, T. K. (1999). The role of phosphatidylinositol 3-kinase in vascular endothelial growth factor signaling. *J. Biol. Chem.* **274**, 10002-10007.

Thomas, M. and Owen, C. (2008). Inhibition of PI-3 kinase for treating respiratory disease: good idea or bad idea?. *Curr. Opin. Pharmacol.* **8**, 267-274.

Vanhaesebroeck, B., Leevers, S. J., Panayotou, G. and Waterfield, M. D. (1997). Phosphoinositide 3-kinases: a conserved family of signal transducers. *Trends Biochem. Sci.* **22**, 267-272.

Wang, S., Li, X., Parra, M., Verdin, E., Bassel-Duby, R. and Olson, E. N. (2008). Control of endothelial cell proliferation and migration by VEGF signaling to histone deacetylase 7. *Proc. Natl. Acad. Sci. USA* **105**, 7738-7743.

Whitman, M., Downes, C. P., Keeler, M., Keller, T. and Cantley, L. (1988). Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate. *Nature* **332**, 644-646.

Wymann, M. P. and Pirola, L. (1998). Structure and function of phosphoinositide 3-kinases. *Biochim. Biophys. Acta* **1436**, 127-150.

Yang, L., Soonpaa, M. H., Adler, E. D., Roepke, T. K., Kattman, S. J., Kennedy, M., Henckaerts, E., Bonham, K., Abbott, G. W., Linden, R. M. et al. (2008). Human cardiovascular progenitor cells develop from a KDR+ embryonic-stem-cell-derived population. *Nature* **453**, 524-528.

Yang, Y., Min, J. Y., Rana, J. S., Ke, Q., Cai, J., Chen, Y., Morgan, J. P. and Xiao, Y. F. (2002). VEGF enhances functional improvement of postinfarcted hearts by transplantation of ESC-differentiated cells. *J. Appl. Physiol.* **93**, 1140-1151.

Yuan, T. L., Choi, H. S., Matsui, A., Benes, C., Lifshits, E., Luo, J., Frangioni, J. V. and Cantley, L. C. (2008). Class 1A PI3K regulates vessel integrity during development and tumorigenesis. *Proc. Natl. Acad. Sci. USA* **105**, 9739-9744.

Zisa, D., Shabbir, A., Suzuki, G. and Lee, T. (2009). Vascular endothelial growth factor (VEGF) as a key therapeutic trophic factor in bone marrow mesenchymal stem cell-mediated cardiac repair. *Biochem. Biophys. Res. Commun.* **390**, 834-838.