Class IA Phosphatidylinositol 3-Kinase Isoform p110α Mediates Vascular Remodeling

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Objective—Neointima formation after vascular injury remains a significant problem in clinical cardiology, and current preventive strategies are suboptimal. Phosphatidylinositol 3′-kinase is a central downstream mediator of growth factor signaling, but the role of phosphatidylinositol 3′-kinase isoforms in vascular remodeling remains elusive. We sought to systematically characterize the precise role of catalytic class IA phosphatidylinositol 3′-kinase isoforms (p110α, p110β, p110δ), which signal downstream of receptor tyrosine kinases, for vascular remodeling in vivo.

Approach and Results—Western blot analyses revealed that all 3 isoforms are abundantly expressed in smooth muscle cells. To analyze their significance for receptor tyrosine kinases–dependent cellular responses, we used targeted gene knockdown and isoform-specific small molecule inhibitors of p110α (PIK-75), p110β (TGX-221), and p110δ (IC-87114), respectively. We identified p110α to be crucial for receptor tyrosine kinases signaling, thus affecting proliferation, migration, and survival of rat, murine, and human smooth muscle cells, whereas p110β and p110δ activities were dispensable. Surprisingly, p110δ exerted noncatalytic functions in smooth muscle cell proliferation, but had no effect on migration. Based on these results, we generated a mouse model of smooth muscle cell–specific p110α deficiency (sm-p110α−/−). Targeted deletion of p110α in sm-p110α−/− mice blunted growth factor–induced cellular responses and abolished neointima formation after balloon injury of the carotid artery in mice. In contrast, p110δ deficiency did not affect vascular remodeling in vivo.

Conclusions—Receptor tyrosine kinase–induced phosphatidylinositol 3′-kinase signaling via the p110α isoform plays a central role for vascular remodeling in vivo. Thus, p110α represents a selective target for the prevention of neointima formation after vascular injury, whereas p110β and p110δ expression and activity do not play a significant role. (Arterioscler Thromb Vasc Biol. 2015;35:1434-1444. DOI: 10.1161/ATVBAHA.114.304887.)

Key Words: neointima ■ p110α ■ p110β ■ phosphatidylinositol 3′-kinase (PI3K) ■ tyrosine kinase ■ vascular remodeling ■ vascular smooth muscle cells

Despite significant improvements in coronary interventions, cardiovascular diseases remain the leading cause of death in developed countries.1 Underlying pathologies, including atherosclerosis and restenosis, after vascular interventions are characterized by pathological remodeling of the arterial wall.2 Neointimal lesions at sites of vascular injury display abnormal intimal accumulation of smooth muscle cells (SMCs), which gradually narrows affected vessels and thus limits blood flow and oxygen supply. The development of drug-eluting stents has led to a significant reduction of restenosis and target-vessel revascularizations.3,4 However, drug-eluting stents have failed to improve mortality, and impaired re-endothelialization, necessitating prolonged dual antiplatelet therapy and resulting in increased rates of stent thrombosis, remains a highly relevant limitation.3,5 Currently used compounds represent rather broad spectrum cell cycle inhibitors, which affect numerous signaling pathways and cell types, including endothelial cells (ECs). Therefore, better characterization of the signal relay mechanisms mediating neointima formation and the development of selective and targeted interventions for its prevention are highly warranted.

Proliferation and migration of SMCs largely contribute to intimal thickening in vascular lesions. Cell division and motility are mainly governed by transmembranous cell surface...
receptors. Although numerous receptors are involved, they share common downstream signaling pathways, providing suitable molecular targets for pharmacological interventions. One of the major pathways leading to cellular proliferation, migration, and survival is relayed by phosphatidylinositol 3'-kinase (PI3K). Previously, we were able to demonstrate that platelet-derived growth factor β receptor (PDGFβR)-dependent activation of PI3K in SMCs promotes cell cycle progression via upregulation of cyclin D1. Prevention of PDGFR-dependent PI3K activation significantly reduced neointima formation after balloon angioplasty, indicating that PI3K is involved in vascular remodeling. In addition to platelet-derived growth factor (PDGF), other growth factors, such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and insulin-like growth factor-1, also contribute to restenosis formation in stented arteries because they promote SMC proliferation, migration, and differentiation. Receptor tyrosine kinases (RTKs), including the PDGFβR, EGFβR, FGFR, and IGFβR, and receptors associated with tyrosine kinase activity signal upstream of class IA PI3K isoforms. These lipid kinases produce phosphatidylinositol-tris-phosphate. Phosphatidylinositol-tris-phosphate serves as a membrane docking site for downstream mediators, such as AKT, which propagates PI3K signaling by phosphorylation of various protein substrates.

Class IA PI3K isoforms are heterodimers consisting of a catalytic subunit (p110α, p110β, p110δ) and a smaller regulatory subunit (p85α, p85β, p85δ). p110α and p110β are ubiquitously expressed, whereas p110δ expression is believed to be restricted to hematopoietic cells. Analysis of mice lacking single p110 isoforms revealed nonredundant and tissue-specific functions of the various p110 isoforms, which raised hope for selective treatment options against disease promoting isoforms with tolerable impacts on physiological needs. As a result, recent efforts have led to the development of isoform-specific small molecule inhibitors, particularly in the field of oncology. However, although recent studies were able to identify relevant PI3K isoforms in cancer, their role in vascular remodeling remains largely elusive.

The main goals of this study were (1) to systematically characterize the specific role of class IA PI3K isoforms for RTK-dependent signaling in SMCs and (2) to establish their significance for vascular remodeling processes in vivo, particularly neointima formation after balloon injury. To achieve these goals, we used a combination of genetic and pharmacological approaches. Although in vitro assays established p110α and, to lesser extent, p110δ to be important for growth factor-dependent SMC proliferation and migration, only p110α deficiency in mice largely suppressed balloon injury-induced neointima formation, whereas p110δ deficiency had no significant impact on restenosis.

Materials and Methods

A detailed description of the Materials and Methods is available in the online-only Data Supplement.

Results

To systematically investigate the role of class IA PI3Ks for neointima formation, we first monitored the expression profile of the 3 catalytic p110 isoforms (p110α, p110β, and p110δ) in rat aortic, as well as in human coronary SMCs. As expected, Western blot analysis revealed that p110α and p110β were abundantly expressed in SMCs (Figure 1A). However, although p110δ expression is believed to be restricted to leukocytes, we surprisingly found that SMCs also express p110δ. Specific siRNA-mediated knockdown of p110 subunits confirmed isoform specificity of the respective antibodies. Additionally, knockdown of specific p110 subunits did not significantly influence the expression of the remaining p110 subunits (Figure 1A). Furthermore, we explored binding of p110 subunits to the activated PDGFβR, representing one of the most potent RTKs in SMCs that largely contributes to neointima formation. The p110 subunits associate via their respective regulatory p85 subunit with specific tyrosine-phosphorylated binding sites within RTK polypeptides. Co-immunoprecipitation experiments using a PDGFβR-specific antibody, subsequent Western blotting for receptor-associated molecules, and densitometric analysis indicated that p110α and p110δ, as well as the regulatory p85 subunit—but not p110β—are recruited to the activated PDGFβR (Figure 1B). This finding was confirmed by immunocytochemical staining (Figure 1C). In resting cells, p110 isoforms are mainly located in the cytoplasm and the perinuclear region. Treatment with PDGF-BB stimulates a partial translocation of p110α and p110β to the cell surface. In contrast, no p110β trafficking was observed on stimulation with PDGF-BB.

Previous studies demonstrated that at least in some cell types, p110β signals downstream of G protein-coupled receptors (GPCRs). We therefore stimulated SMCs with the GPCR agonist lysophosphatidic acid and analyzed the ligand-induced phosphorylation of the PI3K downstream target AKT. Western blot and densitometric analysis presented in Figure 2A demonstrate that lysophosphatidic acid–induced activation of AKT was concentration-dependently inhibited by the p110β inhibitor TGX-221. As expected, TGX-221 did not significantly influence PDGF-induced AKT activation, and although p110β binds to the activated PDGFβR, its catalytic activity seems to be of minor importance because p110β inhibition via IC-87114 had no significant effect. Instead, AKT phosphorylation largely depended on p110α activity because it was completely abrogated by its specific inhibitor PIK-75 (Figure 2B) at concentrations which do not affect

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p110 subunits expression (Figure I in the online-only Data Supplement). Furthermore, inhibition of p110α abrogated mitogenic signaling events downstream of AKT, such as phosphorylation of glycogen synthase kinase-3β (GSK3β), induction of cyclin D1, and phosphorylation of the retinoblastoma protein (Figure 2C). Together, these data indicate that p110α and p110β bind to activated RTKs, such as the βPDGFR in SMCs, and that p110α catalytic activity is mainly responsible for downstream signaling, whereas p110β seems to signal downstream of GPCRs.

**p110α Mediates Smooth Muscle Cell Proliferation, Migration, and Survival**

RTKs, such as the βPDGFR, are mainly involved in cellular proliferation and migration. Therefore, we analyzed the impact of the different p110 subunits on RTK-induced SMC proliferation and chemotaxis by various approaches. Figure 3A demonstrates that the p110α-selective small molecule inhibitor PIK-75 concentration-dependently prevented PDGF-induced SMC proliferation. In contrast, specific inhibition of p110β (TGX-221) and p110δ (IC-87114) was noneffective even at high concentrations (3 μmol/L). Similar results were obtained when SMC proliferation was assessed by FACS analysis, and PIK-75 also inhibited the mitogenic responses of other growth factors, such as EGF and FGF (Figures IIA and IIB in the online-only Data Supplement). Furthermore, inhibition of p110α—but not p110β or p110δ—also abolished PDGF-induced protection of SMCs against H2O2-induced apoptosis (Figure III in the online-only Data Supplement). In a second approach, the contribution of p110 subunits to SMC proliferation was further explored by using specific siRNAs against p110α, p110β, and p110δ, respectively. As expected from the above findings, knockdown of p110α—also abolished PDGF-induced proliferation, whereas suppression of p110β expression had no influence (Figure 3B). However, p110δ knockdown, which sufficiently prevented p110δ recruitment to the activated βPDGFR (Figure IVA in the online-only Data Supplement), surprisingly also impaired SMC proliferation (Figure 3B). Because inhibition of p110δ kinase activity via IC-87114 had no effect even at high concentrations (Figure IVB in the online-only Data Supplement), although the inhibitory potency of IC-87114 on p110δ kinase activity was demonstrated in human monocytes (Figure IVC in the online-only Data Supplement), this suggests a noncatalytic mechanism.
By the use of a second unrelated p110δ-specific siRNA, we were able to confirm this result (Figure IVD in the online-only Data Supplement).

Next, we analyzed the contribution of p110 isoforms to growth factor–induced SMC migration. In 2 distinct experimental approaches, the application of p110 inhibitors indicated that PDGF-mediated chemotaxis of SMCs depended exclusively on the activation of p110α (Figure 3C; Figure V in the online-only Data Supplement). In fact, PIK-75 completely abrogated SMC migration, whereas IC-87114 and TGX-221 had no influence. Consistently, knockdown of p110α by siRNA significantly diminished PDGF-induced chemotaxis, whereas suppression of p110β expression was ineffective (Figure 3D).

In contrast to SMC proliferation, PDGF-induced SMC migration was not affected by p110β knockdown (Figure 3D).

Taken together, analysis of SMC proliferation and chemotaxis clearly demonstrated that growth factors like PDGF exert their mitogenic, migratory, and cell survival responses in SMCs via p110α-dependent pathways.

**p110α Deficiency Profoundly Reduces Neointima Formation In Vivo**

A concern with the use of pharmacological inhibitors is their specificity. Although p110 inhibitors are highly selective among the p110 isoforms, the overall specificity particularly of PIK-75 seems questionable because it also inhibited Erk
specific inhibitors (A) or siRNAs (B). Chemotaxis assay of PDGF-BB-stimulated SMCs in the absence or presence of p110 isoform-specific inhibitors (C) or siRNAs (D). Shown are means±SEM from at least 3 independent experiments. *P<0.05 versus PDGF-BB alone.

Figure 3. Impact of class IA phosphatidylinositol 3′-kinase (PI3K) isoforms for smooth muscle cell (SMC) proliferation and chemotaxis. BrdU incorporation assay of platelet-derived growth factor (PDGF)-BB–stimulated SMCs in the absence or presence of p110 isoform-specific inhibitors (A) or siRNAs (B). Chemotaxis assay of PDGF-BB-stimulated SMCs in the absence or presence of p110 isoform-specific inhibitors (C) or siRNAs (D). Shown are means±SEM from at least 3 independent experiments. *P<0.05 versus PDGF-BB alone.

signaling at least in SMCs (Figure VI in the online-only Data Supplement). To reliably determine the function of p110α-mediated signaling for vascular remodeling in vivo, we therefore aimed to assess the consequences of p110α deficiency on neointima formation after vascular interventions in a genetic model. Because whole body p110α knockout mice or mice expressing a kinase-inactive p110α mutant (p110αD933A/D933A) are not viable and die between E9.5 and E12.5 because of proliferative abnormalities and defects in angiogenesis,19 we generated smooth muscle–specific p110α-deficient mice (sm-p110α−/−). sm-p110α mice were generated using the Cre/floxP system with Cre expressed under the control of the SM22α promoter. In adult tissue, activity of the SM22α promoter is restricted to SMCs, whereas SM22α is expressed transiently in the heart and in skeletal muscle during embryogenesis. The SM22α promoter is not active in nonmuscle cells,20,21 sm-p110α−/− mice are viable and do not display an obvious phenotype under basal conditions. Blood pressure and heart rate are similar to wild-type (WT) controls (Table I in the online-only Data Supplement). There was >53% and >93% reduction of p110α in SMCs isolated from sm-p110α−/− mice and sm-p110α−/− mice, respectively, whereas p110β and p110δ expression remained unchanged, as determined by Western blotting and densitometric analysis (Figure 4A; Figure VII in the online-only Data Supplement). Consistent with the above results using small molecule inhibitors and siRNAs, SMCs isolated from sm-p110α−/− mice did not mediate efficient phosphorylation of AKT, GSK3β, and retinoblastoma protein, as well as induction of cyclin D1 (Figure 4B), and displayed largely reduced proliferative and chemotactic responses to PDGF (Figures 4C and 4D). It is important to note that this effect was not restricted to PDGF because the proliferative responses of SMCs isolated from p110α-deficient mice to other growth factors, such as EGF and insulin-like growth factor-1, were also significantly reduced compared with cells from WT controls (Figure 4E).

To explore the impact of p110α deficiency in SMCs on vascular remodeling in vivo, we conducted balloon angioplasties of left common carotid arteries in sm-p110α−/− and sm-p110α−/− mice. Balloon-triggered vessel injury promotes the migration and proliferation of SMCs in the intimal area. Histochemical analysis of cross sections from the compromised vessels revealed that p110α deficiency in SMCs resulted in robustly decreased neointima formation as compared with the situation in WT animals (Figure 5A). Intimal area (7997±2921 versus 37420±7284 μm², P<0.01) and intima to media ratio (0.34±0.08 versus 0.94±0.19, P<0.01) were significantly reduced (Figures 5B and 5C). In addition, balloon angioplasty induced remodeling in the tunica media because the medial area in WT animals was significantly increased compared with uninjured vessels. In sm-p110α−/− mice, the medial area of uninjured vessels was reduced in comparison to WT animals, and vascular injury did not induce further medial growth (Figure 5D). However, cellularity in the medial area of uninjured vessels did not diverge between sm-p110α−/− (0.0029±0.0001 cells/μm²) and sm-p110α−/− mice (0.0029±0.0002 cells/μm²), suggesting that cell size and extracellular matrix might not be affected by p110α deficiency. PCNA stainings of neointimal sections demonstrated that p110α deficiency nearly completely suppressed
SMC proliferation in vivo (Figures 5E and 5F). There was no difference in the area of adventitia in the injured vessels of sm-p110α−/− and sm-p110α+/+ mice. Western blotting and subsequent densitometric analysis revealed that lack of p110α deficiency and SM22α-positive cells did not significantly contribute to adventitial growth. As expected, the endothelial cell layer in uninjured vessels and re-endothelialized areas in injured vessels were not compromised in sm-p110α−/− mice compared with WT controls (Figure VIII in the online-only Data Supplement). These data are consistent with siRNA-based approaches, indicating again a noncatalytic function of p110α for RTK-induced SMC proliferation. To explore a potential impact of p110α expression on SMC-mediated vascular remodeling in vivo, we conducted balloon angioplasties of left common carotid arteries in p110δ−/− mice. However, histochemical analysis of cross sections from the compromised vessels revealed that p110δ deficiency did not affect intimal area and intima/media ratio and medial and adventitial area compared with WT controls (Figures IXD–IXH in the online-only Data Supplement).

In summary, these data indicate that p110α plays a key role for neointima formation after vascular injury. Despite its effect on SMC proliferation in vitro, p110α expression and activity are of minor importance for vascular remodeling in vivo.

Role of p110 Isoforms in Human Coronary Cells

To evaluate the significance of the above findings for human coronary disease, we also assessed the effects of p110α inhibition by PIK-75 in human coronary SMCs as well as human coronary endothelial cells (hcECs). PDGF-induced proliferation and migration of human coronary SMCs depended on p110α catalytic activity (Figures 6A and 6B). Analysis of apoptosis revealed that under these assay conditions, PIK-75 ≤ 1 μM did not induce apoptosis (data not shown). Furthermore, the inhibitory effect of PIK-75 was not restricted to PDGF because the mitogenic and chemotactic effects of multiple growth factors, including PDGF, FGF, EGF, insulin, and serum, were also inhibited (Figures 6C and 6D).
p110α does not couple to RTKs in SMCs, but rather signals downstream of GPCRs. Although p110α was identified as a crucial target for the prevention of neointima formation, its biological importance may not be restricted to SMCs.

Expression of p110α and p110β in SMCs as assessed herein was as unexpected because previous studies indicated that these isoforms are ubiquitously expressed. In contrast, p110β expression was believed to be restricted to leukocytes, although its expression in SMCs has not been analyzed in detail before. Apart from leukocytes, p110δ expression could not be detected in numerous tissues and cell types, including liver, lung, muscle, and brain, as well as fibroblasts and ECs.22 Therefore, p110δ expression in SMCs appeared unlikely. However, our data provide evidence that this isoform is abundantly expressed in SMCs from human, mouse, and rat origin. Moreover, coimmunoprecipitation experiments demonstrated that p110δ couples to the activated βPDGFR, suggesting a possible function in RTK-induced PI3K signaling. In contrast, p110β—although expressed in SMCs—did not bind to the activated βPDGFR, and its inhibition did not influence PDGF-dependent AKT activation. Consistent with recent studies demonstrating that p110β couples to GPCRs at least in some cell types,18,23 we found that p110β inhibition via TGX-221 abrogated lysophosphatidic acid–induced AKT activation, demonstrating that p110β participates in GPCR

Discussion

Herein, we sought to reveal the importance of class IA PI3K isoforms in SMCs for pathological vascular remodeling. Our data provide strong evidence from genetic models that among RTK inducible class IA PI3K isoforms, only p110α is important for neointima formation and media growth after balloon angioplasty via mediating SMC proliferation and migration. Although p110δ is expressed in SMCs and is recruited to the activated βPDGFR, its catalytic activity is dispensable for SMC proliferation, migration, and neointima formation in vivo. Nevertheless, p110δ seems to exert some noncatalytic functions in RTK signaling. In contrast to p110α and p110δ,
signaling in SMCs. Hence, within class IA PI3K isoforms, p110α and p110δ couple to RTKs in SMCs, whereas p110β seems to signal downstream of GPCR.

Our experiments using isoform-specific p110 inhibitors and gene knockdown by siRNA revealed that p110α is the critical isoform mediating RTK-dependent cell responses in rat, mouse, and human SMCs. This is in accordance with several studies demonstrating a central function for p110α in mitogenic and migratory responses in various cell types, including ECs, retinal cells, intestinal epithelial cells, ovarian cancer cells, and human aortic SMCs. Additionally, the gene encoding p110α (PIK3CA) is one of the most commonly mutated genes identified in numerous human tumors, indicating an important role of p110α in proliferative diseases like cancer, which has led to great efforts to develop isoform-specific PI3K inhibitors. Data presented herein provide further evidence that p110α signaling in SMCs is of major importance during pathological vascular remodeling. Application of PIK-75 resulted in a concentration-dependent inhibition of SMC proliferation with half-maximal effects at 30 to 100 nmol/L in human coronary cells and 100 to 300 nmol/L in rat SMCs. Importantly, these concentrations are relevant in vivo because serum levels ≤117 nmol/L were achieved after systemic application of PIK-75 in mice and appeared sufficient to attenuate pathological remodeling after vessel ligation. Furthermore, our data are consistent with previous publications on tumor proliferation and migration.

**Figure 6.** Effects of p110α inhibition via PIK-75 on cellular responses in human coronary smooth muscle cells (hcSMCs) and human coronary endothelial cells (hcECs). BrdU incorporation (A) and chemotaxis (B) of platelet-derived growth factor (PDGF)-BB–stimulated hcSMCs in the absence or presence of indicated concentrations of PIK-75. BrdU incorporation (C) and chemotaxis (D) of growth factor mixture-stimulated hcSMCs as indicated in the absence or presence of indicated concentrations of PIK-75. BrdU incorporation (E) and chemotaxis (F) of vascular endothelial growth factor (VEGF)–stimulated hcECs in the absence or presence of indicated concentrations of PIK-75. Shown are means±SEM from at least 3 independent experiments. *P<0.05 versus growth factors alone. FCS indicates fetal calf serum.
cell proliferation presenting IC₅₀ values between 19 and 100 nmol/L, whereas lower concentrations were sufficient in cell-free systems. It is well established that PI3K-dependent proliferative responses are mediated through the activation of AKT and that PI3K also contributes to cell migration via activating small GTPases of the Rho family, like Rac, Rho, or Cdc42, which control migratory responses primarily via organization of the actin cytoskeleton. Herein, monitoring of PDGF-induced downstream signaling events in SMCs revealed that the phosphorylation of GSK3β, induction of cyclin D1, and phosphorylation of the retinoblastoma protein depend on p110α kinase activity. These data are in line with previous findings from our laboratory, demonstrating that the βPDGFR induces cell cycle progression of SMCs via PI3K-dependent upregulation of cyclin D1. This is consistent with the concept that AKT-dependent phosphorylation/inactivation of GSK3β leads to upregulation of the cell cycle promoter cyclin D1 by preventing its cytoplasmic relocation and degradation. Furthermore, SMC proliferation stimulated by diverse growth factors like EGF, FGF, or insulin-like growth factor-1 was almost abrogated in these cells, indicating a common function of p110α in growth factor–induced cell cycle progression. Consequently, intimal area was robustly reduced in sm-p110α/− mice compared with WT controls 28 days after injury. Simultaneously, intimal cellular proliferation was almost abrogated in these mice as measured by PCNA staining. Because there is no evidence that cell cycle progression of SMCs is differentially regulated at different time points after injury and considering an almost 80% reduced intimal area in injured sm-p110α/− mice, one must assume that lack of p110α expression should also impair SMC proliferation at early time points. Taken together, these data indicate that p110α is crucial for growth factor–induced cell cycle progression in vitro and in vivo.

When identifying signaling pathways and molecular targets, a concern with the use of pharmacological inhibitors is their specificity. In particular, although PIK-75 is highly selective among the p110 isoforms, its overall specificity seems questionable (see Figure VI in the online-only Data Supplement). Therefore, it is important to note that we obtained similar results by using 3 different approaches, namely inhibition of p110α kinase activity by PIK-75, gene knockdown by siRNA, and targeted deletion of p110α in SMCs. To evaluate the importance of p110α for neointima formation in vivo, we generated SMC-specific p110α knockout mice. sm-p110α/− mice were viable, underwent normal development, and had no obvious phenotype under basal conditions, but showed reduced medial area in carotid arteries compared with their WT littermates. This suggests that p110α as a common downstream mediator of multiple RTKs might affect normal vessel development most likely via mediating SMC proliferation. After balloon injury, lack of p110α in SMCs largely reduced neointima formation. Our data that were obtained by a genetic model using sm-p110α/− mice are consistent with a recent study demonstrating that pharmacological inhibition of p110α by systemic administration of PIK-75 selectively interfered with arterial thrombosis and neointima formation in a ligation model of vascular injury. However, although these investigators reported that p110α inhibition does not affect EC function and re-endothelialization after vascular injury, we found that VEGF-dependent proliferation and migration of hcECs are also markedly impaired by PIK-75. The latter finding is in accordance with recent genetic studies demonstrating that p110α is essential for EC migration, proliferation, and survival and that EC-specific p110α deficiency led to embryonic lethality because of severe defects in vascular development. This discrepancy may be explained by differences in cells (HAEC versus hcEC), by different concentrations of PIK-75, and by the fact that in the study by Holy et al., which also activates GPCRs—was used as an inducer of EC responses, which may potentially override RTK-mediated effects. Our results show that hcEC proliferation and migration on stimulation with VEGF, representing the most relevant growth factor for ECs that regulates vascular development and endothelial repair mechanisms, were concentration-dependently inhibited by PIK-75. Although we were not able to analyze the effects of p110α on re-endothelialization in vivo because EC-specific p110α-deficient mice are not viable, this finding may have important implications because inhibition of VEGF-dependent EC responses is believed to ameliorate proper endothelial coverage after vascular injury. On the other hand, SMC-specific ablation of p110α led to an almost complete reduction of neointima formation after vessel injury without affecting re-endothelialization.

Thus, our results indicate that cell type–specific interference with p110α signaling in SMCs may be required to use this pathway as an attractive approach to robustly reduce neointima formation without affecting proper re-endothelialization.

Although p110δ also binds to the activated βPDGFR, its function remains obscured. On the one hand, lack of p110δ expression significantly diminished PDGF-dependent proliferation, as shown by siRNA-mediated knockdown of p110δ in rat SMCs, and in cells isolated from p110δ-deficient mice. On the other hand, p110δ catalytic activity seems dispensable for SMC proliferation and migration. These data suggest a kinase-independent function of p110δ, as previously demonstrated for p110β. Expression of kinase-inactive p110β rescued the impaired proliferation of mouse embryonic fibroblasts lacking p110β and also restored internalization of transferrin and activated EGFR, suggesting a kinase-independent function of p110β in endocytosis, which is required for cell proliferation. Although these data indicate that a potential scaffolding function on cell proliferation is not restricted to p110δ, the underlying noncatalytic mechanisms of class IA p110 subunits remain elusive. Whether kinase-independent actions of p110δ are relevant in physiological or pathological processes is not yet clear. At least during neointima formation after balloon angioplasty, p110δ deficiency seems to be of minor importance, although it diminished SMC proliferation in vitro. This may be explained by the fact that numerous cellular responses, including mitogenic, migratory, and survival signals, are required for neointima formation.

In summary, the data presented herein provide a systematic characterization of the role of class IA PI3K isoforms for cell proliferation, migration, and survival of SMCs, representing
highly relevant processes during vascular remodeling. By using a genetic model, p110α was identified as the critical isoform mediating these cellular events, and its lack in SMCs completely abrogated neointima formation after balloon injury in sm-p110α−/− mice. However, cell type–specific targeting of p110α may be required because VEGF-dependent responses of hECs are also mediated via this isoform.

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Disclosures

None.

References


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Our data demonstrate that among class IA phosphatidylinositol 3′-kinase isoforms, p110α is crucial for cell proliferation, migration, and survival of smooth muscle cells. Consequently, lack of p110α expression in smooth muscle cells abrogated neointima formation after balloon injury in sm-p110α−/− mice in vivo. These data provide novel insights into a role for class IA phosphatidylinositol 3′-kinase isoforms in vascular remodeling and indicate that p110α represents a selective target for the prevention of neointima formation after vascular injury, whereas p110β and p110δ expression and activity do not play a significant role.
Supplementary Figure I. A. Western blot analysis of p110 protein expression in PDGF-BB (24 h) stimulated SMCs. p110-specific inhibitors (LY294002: pan-PI3K; PIK-75: p110α; TGX-221: p110β; IC-87114: p110δ) were added as indicated. α-Actin is shown as a loading control. B. Densitometric analysis of three independent experiments.
Supplementary Figure II. Impact of p110α inhibition on growth factor-induced SMC proliferation. A. SMC proliferation as assessed by FACS analysis. Data represent cells in S phase. B. BrdU incorporation in unstimulated and EGF- or FGF-stimulated SMC in the presence of indicated concentrations of LY (PI3K), PIK-75 (p110α), TGX-221 (p110β), IC-87114 (p110δ), or 15e (p110α). Shown are means ± SEM from at least three independent experiments. *p<0.05 versus growth factor alone.
**Supplementary Figure III.** Impact of class IA PI3K isoforms on growth factor-dependent protection from SMC apoptosis. Apoptosis of SMCs was assessed by Cell Death Detection ELISA of PDGF-BB stimulated SMCs in the absence or presence of the pan PI3K inhibitor LY294002, or isoform-specific inhibitors of p110α (PIK-75), p110β (TGX-221), and p110δ (IC-87114) as indicated. Data represent means ± SEM from at least three independent experiments. *p<0.05 versus H₂O₂ and PDGF-BB.
**Supplementary Figure IV. Role of p110δ in SMCs and effectiveness of p110δ inhibitor in SMCs and monocytes.**

**A.** Co-immunoprecipitation of the unstimulated or activated (50 ng/ml PDGF-BB) βPDGFR with p110α, p110δ, and p85 in SMCs, demonstrating that p110δ knockdown by siRNA sufficiently prevented p110δ binding to the βPDGFR. Western blot analysis of AKT phosphorylation in PDGF-BB-stimulated SMCs. p110δ-specific inhibitor IC-87114 was added at indicated concentrations. AKT is shown as a loading control. Densitometric analysis is shown above.

**B.** Positive control for IC-87114: Representative Western blot demonstrating AKT activation of unstimulated, insulin-, or M-CSF-stimulated human monocytes in the absence or presence of the pan PI3K inhibitor LY294002 (20 μM) or the p110δ inhibitor IC-87114 (1 μM). p110δ and β-actin blots are shown as loading controls. BrdU incorporation of PDGF-BB stimulated SMCs in the absence or presence of p110δ specific siRNAs. Shown are means ± SEM from at least three independent experiments. *p<0.05 versus n.s. siRNA control.
**Supplementary Figure V.** *Impact of isoform-specific p110 inhibitors on PDGF-induced migration of SMCs.* Scratch assay demonstrating SMC migration in response to PDGF-BB (10 ng/ml) from the scratch edge at times indicated. Inhibitors of p110α (PIK-75; 1 µM), p110β (TGX-221; 3 µM), and p110δ (IC-87114; 3 µM) were applied 60 min before PDGF stimulation as indicated.
Supplementary Figure VI. Impact of isoform-specific p110 inhibitors on AKT and ERK (p42/44) phosphorylation in PDGF-BB (50 ng/ml) stimulated SMCs. Pan PI3K inhibitor LY294002 and isoform-specific inhibitors of p110α (PIK-75), p110β (TGX-221), and p110δ (IC-87114) were added as indicated. Shown are representative Western blots for p-ERK, p-AKT and RasGAP (loading control).
<table>
<thead>
<tr>
<th></th>
<th>Heart Rate [beats/min]</th>
<th>SBP sys. [mmHg]</th>
<th>SBP dia. [mmHg]</th>
<th>MAP [mmHg]</th>
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</thead>
<tbody>
<tr>
<td>sm-p110α&lt;sup&gt;+/+&lt;/sup&gt; (n=8)</td>
<td>435.9±10.6</td>
<td>96.8±2.7</td>
<td>70.6±2.2</td>
<td>79.4±2.4</td>
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<tr>
<td>sm-p110α&lt;sup&gt;-/-&lt;/sup&gt; (n=8)</td>
<td>419.2±12.4</td>
<td>94.3±3.3</td>
<td>62.8±2.7</td>
<td>73.3±2.8</td>
</tr>
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</table>

**Supplementary Table I.** Heart rate, systolic blood pressure (SBP sys), diastolic blood pressure (SBP dia), and mean arterial blood pressure (MAP) from sm-p110α<sup>+/+</sup> and sm-p110α<sup>-/-</sup> mice. Blood pressure was invasively measured under resting conditions.
Supplementary Figure VII. Densitometric analysis of p110 subunit expression in mSMCs from sm-p110α+/+, sm-p110α−/−, and sm-p110α−/− mice. Data were normalized to α-actin control and expressed as percentage of p110 expression in sm-p110α+/+ cells. Densitometric analysis were performed of three independent experiments (p<0.01, p<0.000005 vs. WT).
Supplementary Figure VIII. Re-endothelialization and endothelial cell layer are comparable in sm-p110α+/+ and sm-p110α−/− mice. Representative histological cross-sections (immunohistochemical van Willebrand factor staining) of uninjured and injured common carotid arteries from sm-p110α−/− mice and littermate WT controls (sm-p110α+/+), 4 weeks after balloon injury.
Supplementary Figure IX. Role of p110δ for cellular responses of SMCs and neointima formation following balloon angioplasty. A. Expression of p110 isoforms in isolated SMCs from p110δ+/- and p110δ-/- mice; B. BrdU incorporation; C. Chemotaxis. SMCs were isolated from p110δ+/- and p110δ-/- mice and stimulated with buffer or PDGF-BB as indicated. *p<0.05 versus unstimulated control. Shown are means ± SEM from at least three independent experiments. *p<0.05 versus p110δ+/. D. Representative histological cross-sections (hematoxylin eosin staining) of uninjured and injured common carotid arteries from male p110δ-/- mice and littermate WT controls (p110δ+/-), 3 weeks after balloon injury. E. Intimal area; F. Intima-to-media ratio (I/M) of carotid arteries from p110δ+/- (n=6) and p110δ-/- mice (n=8), 3 weeks after balloon injury. G. Adventitial area of carotid arteries from p110δ+/- and p110δ-/- mice. H. Medial area of uninjured (left) and injured (right) carotid arteries from p110δ+/- and p110δ-/- mice.
Supplementary Figure X. Densitometric analysis of p110 subunit expression in mSMCs from p110δ+/+ and p110δ−/− mice. Data were normalized to α-actin control and expressed as percentage of p110 expression in p110δ+/+ cells. Densitometric analysis were performed of three independent experiments (#p<0.0001 vs WT).
On-line Supplementary Material

Analysis of Class IA Phosphatidylinositol 3-kinase Isoforms in Smooth Muscle Cells Reveals that only p110α Is Important for Vascular Remodeling

Marius Vantler, Joana Jesus, Olli Leppänen, Maximilian Scherner, Eva Maria Berghausen, Xin Chen, Lenard Mustafov, Tilmann Kramer, Mario Zierden, Maximilian Gerhardt, Henrik ten Freyhaus, Florian Blaschke, Anja Sterner-Kock, Stephan Baldus, Jean J. Zhao, Stephan Rosenkranz

Materials and Methods

Generation of smooth muscle-specific p110α deficient mice

Smooth muscle specific p110α knockout mice were generated by cross-breeding of homozygous p110α^{flox/flox} mice (detailed in\(^1\), C57BL/6 background) with SM22-Cre mice\(^2\) (B6.Cg-Tg(Tagln-cre)1Her/J, C57BL/6 background, Jackson Laboratory) which express Cre recombinase under the control of the mouse transgelin (smooth muscle protein 22-alpha) promoter. SM22-Cre\(^{-/-}\)/p110α^{flox/+} mice were crossed to homozygous p110α^{flox/flox} mice to generate SM22-Cre\(^{-/-}\)/p110α^{flox/flox} (sm-p110α\(^{-/-}\)), SM22-Cre\(^{+/+}\)/p110α^{flox/+} (sm-p110α\(^{+/+}\)) and SM22-Cre\(^{+/+}\)/p110α^{flox/flox} (sm-p110α\(^{+/+}\)) littermates. Mice were born at normal Mendelian ratio, developed normally without any obvious phenotype under physiological conditions, and displayed a normal life expectancy.

Animals and Diets

sm-p110α\(^{-/-}\), sm-p110α\(^{+/+}\), sm-p110α\(^{+/+}\) and p110δ\(^{-/-}\) mice and WT littermates (kindly provided by Dr. Roland Piekorz, Universitätsklinikum Düsseldorf, Germany)\(^3\) were kept on a regular diet until experimentation and fed a chow diet (D12108 containing 1.25% cholesterol, Research Diets, New Brunswick, NJ) that was started 5 days prior to intervention and continued until harvesting. All animal experiments were performed in accordance with institutional guidelines and approved by the local animal committee.

Assessment of blood pressure and heart rate

Mice were anesthetized by a mixture of compressed air (0.6 l/min), oxygen (1-1.2 l/min) and Isoflurane (2%) through gas dissipating respiratory mask. Body temperature of animals was regulated using a rectal thermometer-connected heating plate. The systemic blood pressure and heart rate were monitored in the left carotid artery using a millar® catheter (Mil-SPR-1000). The catheter information was amplified by a PowerLab® amplifier and processed using LabChart7® software (ADinstruments). Mean arterial blood pressure (MAD) was approximated from systolic blood pressure (SBP sys) and diastolic blood pressure (SBP dia): MAD = SBP dia + 1/3 x (SBP sys – SBP dia).

Mouse Carotid Balloon Injury

Balloon injury of the left proximal common carotid artery was performed in male WT control, sm-p110α\(^{-/-}\), and p110δ\(^{-/-}\) mice (10-14 weeks of age) as described\(^4\).
Briefly, animals were anaesthetized with isoflurane (1.5%), the left carotid bifurcation was dissected, and the mid-common carotid artery was dilated to approximately 1.3 x the unmanipulated mid-common carotid artery diameter for 60 seconds, using a miniature angioplasty catheter (Schwager Medica, Winterthur, Switzerland; balloon-length 5 mm, diameter range: 0.72 mm at 8 bars to 0.83 mm at 16 bars) that was introduced through proximal external carotid artery cut down. After deflation, the catheter was removed, the external carotid artery ligated and antegrade flow was re-established to the internal carotid artery. p110δ mice were euthanized 21 days after balloon injury. Since neointima formation was barely detectable in the sm-p110α-/− mice, we prolonged the post-injury period of p110α animals to 28 days in order to facilitate the measurement of the intimal area in the sm-p110α-/− mice.

Tissue Harvesting and Processing

After puncturing the left ventricle and cutting the right atrium, vessels were rinsed with phosphate-buffered saline (PBS) and perfusion-fixed at approximately 100 mmHg with 20 ml of 4% paraformaldehyde (Santa Cruz Biotechnology, USA) in PBS. The injured left and the uninjured right common carotid artery were excised after dissecting the adventitia. After fixation with 4% paraformaldehyde overnight, the vessels were embedded in paraffin blocks and processed further.

Morphometric and histological analysis

Following paraffin embedding of the tissue, three serial cross-sections (5 µm thickness, 1000 µm apart) were taken from the mid-portion of the dilated segment for histomorphological analysis. Corresponding sections were obtained from the uninjured right common carotid artery. Morphometric analyses were performed using Image J analysis software (NIH). The mean intima/media ratio was calculated in triplicates for each mouse by determining the area surrounded by the internal elastic lamina, the area surrounded by the external elastic lamina, and the lumen area. Nuclear staining was performed with Harries hematoxylin following standard procedures.

Proliferating cells at the balloon injury sites were detected via proliferating cell nuclear antigen (PCNA) staining. Briefly, deparaffinized sections were incubated overnight with primary antibody (rabbit anti-PCNA, Cell signaling #13110, 1:500). PCNA positive nuclei were detected using IHC-Kit DCS SuperVision 2 HRP (#PD000POL, DCS Innovative Diagnostik-Systeme, Germany) according to the manufacturer’s specifications. Mean fraction of PCNA-positively stained nuclei versus hematoxylin positive nuclei were calculated for each mouse in triplicates.

Endothelial cells in injured and uninjured vessels were detected using van Willebrand factor antibody (Dako, # A0082, Germany) and ImmPRESS Reagent Kit, Anti-rabbit (Vector Laboratories, USA), according to the manufacturer’s specifications.

Cell Culture and treatment with siRNAs.

Rat and mouse SMCs were isolated from thoracic aorta by enzymatic dispersion as described5. Animals were sacrificed and the aorta was dissected, removed, and incubated with 1 ml enzyme solution (1 mg/ml Collagenase Type I, 0.3 mg Elsatase, 0.3 mg Trypsin inhibitor in DMEM / 20% FBS) for 15 minutes to detach the adventitia. The remaining adventitia was removed using anatomic forceps, and the endothelium was gently removed by scraping the luminal surface. The aorta was cut into smaller pieces and incubated for further 90 minutes at 37°C in the enzyme solution to disintegrate the tissue and separate the SMCs. After centrifugation, the cell pellet
was resuspended in DMEM culture medium containing 20% FCS and 1% Penicillin/Streptomycin. Cells were transferred into a well of a 24 well plate. After 2 days most of the cells were attached to the plate. Culture medium was changed after three days. After reaching 80% confluence, cells were expanded.

SMCs were grown in a 5% CO₂ atmosphere at 37°C in DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 1% nonessential amino acids (100X), and 10% FCS. Human coronary smooth muscle cells (hcSMCs) and human coronary endothelial cells (hcECs) were obtained from Lonza (Basel, Switzerland). hcSMCs were cultured in Clonetics SmBm and hcECs in EBM-2 media in accordance with the manufacturer’s instructions. Experiments were performed with cells from passages 5 to 10.

siRNA transfections were performed according to the manufacturer’s instructions (Lipofectamine RNAiMax, Invitrogen). Briefly, one day before transfection, cells were plated in 6-well (for Western blotting or chemotaxis assays) or 96-well plates (for proliferation assays) such that they will be 30% confluent at the time of transfection. p110 specific siRNAs (p110α: Rn_Pik3ca_1 FlexiTube siRNA SI02989721, p110β: Rn_Pik3cb_2 FlexiTube siRNA SI01960882, p110δ: Rn_LOC366508_1 FlexiTube siRNA SIO1883007, Rn LOC366508_2 FlexiTube siRNA SIO1883014, Qiagen, Hilden, Germany) and non-silencing control siRNA (#1022076, Qiagen, Hilden, Germany) were used at 10 nM to transfect SMCs in Opti-MEM-I medium using Lipofectamine RNAiMax (Invitrogen). 72 h following transfection, cells were subjected to proliferation and chemotaxis assays or Western blot analysis, respectively.

Immunoblotting, co-immunoprecipitation

Quiescent SMCs were left resting or stimulated with 50 ng/ml PDGF-BB for times indicated. Inhibitors were applied 60 min before PDGF stimulation. Cells were washed twice with HS (20 mm Heps, pH 7.4, 150 mm NaCl), and then lysed in EB (10 mm Tris-HCl, pH 7.4, 5 mm EDTA, 50 mm NaCl, 50 mm NaF, 1% Triton X-100, 0.1% bovine serum albumin, 20 µg/ml aprotinin, 2 mm Na₃VO₄, 1 mm phenylmethylsulfonyl fluoride). Lysates were centrifuged (20 min, 12,000 × g), and the supernatants were subjected to Western blot analysis. For immunoprecipitation, SMCs were stimulated with PDGF-BB (50 ng/ml, 5 min) or left untreated. The βPDGFR was precipitated with a polyclonal βPDGFR antibody (97A) detecting the kinase insert (amino acids 698-797) of the human βPDGFR. βPDGFR immunoprecipitations representing approximately 3 × 10⁶ cells were resolved on a 7.5% SDS-polyacrylamide gel, and the proteins were transferred to PVDF membrane (BioRad Laboratories, USA) and subjected to immunoblot analysis. To monitor the association of signaling molecules with the activated βPDGFR, receptor immunoprecipitations from resting or PDGF-stimulated cells were subjected to Western blot analysis using antisera against human βPDGFR, phospho-βPDGFR (Tyr 751), p110α, p110β, p110δ, and p85. Western blots were subjected to densitometric analysis using Adobe Photoshop CS4 software. Data were normalized to respective loading controls (AKT, GSK3β, actin, RasGAP).

Immunofluorescence

Rat vascular smooth muscle cells were grown on coverslips to 40-50% confluence in DMEM medium with 10% fetal calf serum. Cells were serum starved for 24 hours and thereafter treated with vehicle or 50 ng/ml PDGF-BB for 5 min. After treatment, the cells were washed twice in ice-cold PBS and fixed in 4% paraformaldehyde for 15min at room temperature. The cells were then washed with PBS again and were
permeabilized in 0.5% Triton X-100 for 15min. After blocking in 5% goat serum (Sigma Aldrich, CA, USA) cells were incubated with antibodies against the p110 isoforms p110α (#4249, Cell Signaling, MA, USA), p110β (S-219, Santa Cruz Biotechnology, CA, USA) and p110δ (H-219, Santa Cruz), respectively. Parallel immunostaining was performed using a phospho-PDGFR antibody (pY857, Santa Cruz). Incubation was performed while gently shaking overnight at 4°C. The following day, cells were washed in PBS and incubated with a Cy3 (red) fluorochrome-conjugated 2nd Antibody (Jackson Immunoresearch, PA, USA) for 1 hour at RT. Cells were mounted with a DAPI (blue) containing mounting media (Prolong Gold Antifade Reagent with DAPI, Molecular Probes, CA, USA). 8 bit grayscale images at 300 pixels per inch resolution were acquired using a fluorescence microscope (Keyence Biorevo BZ-9000, Japan). Images were pseudocolored red for p110 antibodies and PDGFR antibody, both probed with Cy3-conjugated secondary antibody, and blue for DAPI nuclear stain. Images are representative of 3 independent experiments, and in each experiment 3 random fields were photographed with oil immersion at 60X 2.5X magnification. Images were imported in Adobe Photoshop CS6 for uniform background subtraction and multichannel images were then merged using NIH ImageJ (https://imagej.nih.gov). Scale bars represent 20um. For presentation purposes contrast and brightness were enhanced uniformly.

Chemotaxis and scratch assays
PDGF-dependent chemotaxis was assayed utilizing a 48-well modified Boyden chemotaxis chamber (NeuroProbe Inc., Baltimore,MD) and collagen-coated polycarbonate, PVDF, filters (8 µm pore size) (GE Water & Process Technologies, Watertown, MA, USA) as described7. Briefly, the lower wells of the chamber were filled with DMEM supplemented with 10 ng/ml PDGF-BB or vehicle in the presence or absence of inhibitors as indicated. The filters were coated with 50 mg/ml rat type I collagen (BD Bioscience, Bedford, MA, USA) and fixed atop the bottom wells. SMCs were trypzined and diluted in DMEM to a final concentration of 4 x 10^5 cells per ml. Where indicated, cells were incubated for 60 min with inhibitors and placed into the upper chamber (50 µl per well). The chamber was kept for 5 h at 37°C in a 5% CO₂ atmosphere and was then disassembled. The cells on the upper surface of the filter were removed, and the cells on the lower surface were fixed and stained with Diff-Quick (Baxter Healthcare, Miami, FL). For quantification, cells on the lower surface of the filter in each well were counted using a 20x magnification raster ocular.

In order to analyze SMC migration, a scratch assay was performed. SMCs were grown to 90% confluency and serum-starved overnight in serum free conditions. The monolayer was scratched to generate a cell free area. Where indicated, SMCs were stimulated with inhibitors 60 min before PDGF-BB (10 ng/ml) application. Cells were allowed to migrate for times indicated.

DNA synthesis assays
DNA synthesis was measured by a 5-bromodeoxyuridine (BrdU)-incorporation assay. 10,000 cells per well were cultured in 96-well plates in DMEM containing 10% FCS, washed with PBS, starved in DMEM for 24 h, and stimulated with PDGF-BB (50 ng/ml), bFGF (50 ng/ml), EGF (50 ng/ml), IGF-I (30 ng/ml), or VEGF (50 ng/ml) for 18 h in the presence or absence of inhibitors as indicated. BrdU incorporation was carried out according to the manufacturer’s specifications (Roche) with an incubation time of 5 h as previously described7. Additionally, SMCs were analyzed following fixation with 70% ethanol (Sigma) by staining with propidium iodide (10
µg/ml, Sigma) in PBS buffer containing 0.25 % TritonX-100 (Sigma) and RNase A (10 µg/ml, Sigma). Cell cycle progression was further assessed by FACS analysis using a FACSCanto I/II (BD Bioscience).

**Apoptosis assay**

A Cell Death Detection ELISA (Roche) was used to determine cell death. The method relies on the detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) that are generated in apoptotic cells. Rat SMCs (10,000 cells) were plated in DMEM containing 10% FCS. 24 h hours later, cells were starved in serum free DMEM for 24 h. To induce apoptosis, medium was changed to DMEM containing 100 µM H₂O₂ in the presence or absence of 50 ng/ml PDGF-BB. Where indicated, cells were stimulated with p110 isoform-specific inhibitors 60 min prior to PDGF application. After 16 h, the cell lysate was collected, and the amount of cell death was evaluated using the Cell Death Detection ELISA assay as described in detail by the manufacturer.

**Materials, antibodies, growth factors, inhibitors, and media**

Chemicals were obtained from Sigma-Aldrich Chemie GmbH, (Steinheim, Germany). Rat tail Collagen type I was from BD Biosciences, Elastase from Serva Electrophoresis GmbH, (Heidelberg, Germany), and Collagenase from Sigma-Aldrich Chemie GmbH, (Steinheim, Germany). The anti-phosphotyrosine antibodies were purchased from Santa Cruz (PY20) and Upstate Biotechnology (4G10). The antibody against RasGAP (69.3) and the βPDGFR (97A) were kind gifts from Andrius Kazlauskas (Harvard Medical School, Boston, USA). The phospho-Akt (Ser473 #4060, Thr308 #13038), phospho-ERK1/2 (#9101), p85 (#4292), p110α (#4255), p110β (#3011) and PCNA (#13110) antibodies were from Cell Signaling technologies (USA), the p110δ (sc-7176) and Muscle actin (#sc-53141) antibodies from Santa Cruz (USA). PDGF-BB, bFGF, EGF, and IGF-I were purchased from Promo Cell (Heidelberg, Germany). LY-294002 and PIK-75 were from Merck (Darmstadt, Germany), TGX-221 from Axxora (New York, USA), and IC-87114 from Symansis (Oackland, New Zealand). Compound 15e was a kind gift from Peter R. Shepherd, University of Auckland, New Zealand). DMEM (High glucose (4.5g/l) with L-Glutamine) was obtained from PAA Laboratories GmbH (Pasching, Austria) and Clonetics SmBm and EBM-2 media were from Lonza (Basel, Switzerland). Fetal calf serum (FCS) was from Gibco, Invitrogen (Carlsbad CA, USA).

**Statistical Analysis**

All data are expressed as means ± SEM. Statistical significance of differences was calculated using ANOVA with post hoc Tukey test or Student unpaired t test. P<0.05 was considered statistically significant.

**References**

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