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J Am Coll Cardiol. Author manuscript; available in PMC 2013 August 02.

Published in final edited form as:
J Am Coll Cardiol. 2011 June 21; 57(25): 2527–2538. doi:10.1016/j.jacc.2011.02.037.

Disruption of Platelet-Derived Growth Factor–Dependent Phosphatidylinositol 3-Kinase and Phospholipase Cγ 1 Activity Abolishes Vascular Smooth Muscle Cell Proliferation and Migration and Attenuates Neointima Formation In Vivo

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Abstract

Objectives—We tested the hypothesis whether selective blunting of platelet-derived growth factor (PDGF)–dependent vascular smooth muscle cell (VSMC) proliferation and migration is sufficient to prevent neointima formation after vascular injury.

Background—To prevent neointima formation and stent thrombosis after coronary interventions, it is essential to inhibit VSMC proliferation and migration without harming endothelial cell function. The role of PDGF—a potent mitogen and chemoattractant for VSMC that does not affect endothelial cells—for neointima formation remains controversial.

Methods—To decipher the signaling pathways that control PDGF beta receptor (βPDGFR)–driven VSMC proliferation and migration, we characterized 2 panels of chimeric CSF1R/βPDGFR mutants in which the binding sites for βPDGFR-associated signaling molecules (Src, phosphatidylinositol 3-kinase [PI3K], GTPase activating protein of ras, SHP-2, phospholipase Cγ 1 [PLCγ]) were individually mutated. Based on in vitro results, the importance of PDGF-initiated signals for neointima formation was investigated in genetically modified mice.

Results—Our results indicate that the chemotactic response to PDGF requires the activation of Src, PI3K, and PLCγ, whereas PDGF-dependent cell cycle progression is exclusively mediated by PI3K and PLCγ. These 2 signaling molecules contribute to signal relay of the βPDGFR by

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The authors have reported that they have no relationships to disclose.

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differentially regulating cyclin D1 and p27[kip1]. Blunting of βPDGFR-induced PI3K and PLCγ signaling by a combination mutant (F3) completely abolished the mitogenic and chemotactic response to PDGF. Disruption of PDGF-dependent PI3K and PLCγ signaling in mice expressing the F3 receptor led to a profound reduction of neointima formation after balloon injury.

Conclusions—Signaling by the activated βPDGFR, particularly through PI3K and PLCγ, is crucial for neointima formation after vascular injury. Disruption of these specific signaling pathways is sufficient to attenuate pathogenic processes such as vascular remodeling in vivo.

Keywords

PI-3 kinase; PLCγ; platelet-derived growth factor; proliferation; restenosis

Despite novel treatment options to achieve vessel patency after percutaneous coronary interventions, vascular restenosis remains a significant clinical problem (1). Critical events for neointima formation at sites of vascular injury are the attraction of vascular smooth muscle cells (VSMCs) from the media, their neointimal proliferation, and extracellular matrix deposition (1–3). The inhibition of central cell cycle–regulating proteins such as mammalian target of rapamycin is efficient in reducing neointima formation after coronary stenting (4,5). However, this approach is hampered by the burden of in-stent thrombosis, because mammalian target of rapamycin inhibition also restrains the proliferation of endothelial cells and thus attenuates re-endothelialization (6,7). Sophisticated novel therapeutic approaches should selectively target VSMC proliferation and migration without affecting endothelial cell function.

Platelet-derived growth factor (PDGF) represents the most potent mitogen and chemoattractant for VSMCs (8,9), whereas it does not elicit significant effects on endothelial cells. Furthermore, PDGF and its beta receptor subtype (βPDGFR), which is abundantly expressed in VSMC (10,11), are significantly upregulated and activated at sites of vascular injury (12,13), making the βPDGFR a promising target for therapeutic interventions. However, recent studies on the role of the βPDGFR for neointima formation yielded conflicting results (14,15). Although inhibition of PDGFR signaling was effective in reducing restenosis formation in animal models, systemic administration of a PDGFR inhibitor failed to prevent the recurrence of in-stent restenosis in humans. Therefore, it appears crucial to more precisely define the role of PDGF for neointima formation and to identify the signaling pathways by which the βPDGFR mediates cellular responses that contribute to vascular disease. We used a genetic approach to address 3 important questions: 1) what are the signaling pathways that mediate PDGF-dependent migration and proliferation in VSMCs; 2) is βPDGFR signaling really critical for neointima formation in vivo; and 3) can the disruption of specific signaling pathways prevent neointima formation? Upon ligand binding, the βPDGFR recruits and activates several SH2 domain-containing signaling molecules that each stably associate with the receptor at phosphorylated tyrosine residues that serve as specific docking sites (16). These include Src family kinases (Src), phosphatidylinositol 3-kinase (PI3K), the GTPase-activating protein of Ras (RasGAP), SHP-2, and phospholipase Cγ1 (PLCγ). To systematically investigate the role of each individual signaling pathway, we used a series of chimeric colony-stimulating factor-1 receptor (CSF1R)/βPDGFR mutants in which the tyrosine residues required for binding of each signaling molecule were individually replaced with phenylalanine (17). Using this system, we were able to precisely characterize the signal relay mechanisms that govern PDGF-dependent chemotaxis and cell cycle progression in VSMCs. These studies particularly identified PI3K and PLCγ as critical signaling enzymes in vitro. Consequently, blunting of PDGF-dependent PI3K and PLCγ signaling in F3 mice, which lack the

respective binding sites in the βPDGFR (18), dramatically reduced neointima formation after vascular injury in vivo.

Methods

Generation of chimeric CSF1R/βPDGFR mutants

The chimeric receptor (ChiR) cDNA was constructed from DNA encoding amino acids 1 to 513 of the human CSF1R and amino acids 528 to 1,106 of the human βPDGFR joined at an EcoRI site (19). Mutations in the βPDGFR sequence are as described (17).

Animals and diets

βPDGFR-F3 mice were generated by introducing point mutations into the βPDGFR locus by gene targeting as described (18). Male $\beta PDGFR^{F3/F3}$ mice and littermate wild-type (WT) controls (C57BL/6J) were kept on a regular diet until experimentation. Mice were fed a chow diet (D12108 containing 1.25% cholesterol; Research Diets, New Brunswick, New Jersey) that was started 5 days before intervention and continued until harvesting. All animal experiments were performed in accordance with institutional guidelines and approved by the local animal committee.

Mouse carotid balloon injury

Balloon injury of the left proximal common carotid artery was performed in WT and $\beta PDGFR^{F3/F3}$ mice (10 to 14 weeks of age) as described (20). Briefly, the mid-common carotid artery was dilated to approximately 1.3 times the unmanipulated diameter for 60 s, using a miniature angioplasty catheter (Schwager Medica, Winterthur, Switzerland). Mice were euthanized 21 days after balloon injury. Vessels were perfusion fixed with 4% paraformaldehyde. The injured left and the uninjured right common carotid artery were excised. After post-fixation with 4% paraformaldehyde overnight, the vessels were embedded in paraffin blocks and processed further.

Morphometric analysis

Three serial cross-sections (5-μm thickness, 1,000 μm apart) were taken from the midportion 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 (National Institutes of Health, Bethesda, Maryland).

Immunoblotting

Quiescent VSMCs were left resting or stimulated with 50 ng/ml of PDGF-BB or macrophage colony-stimulating factor (M-CSF) for times indicated in the presence or absence of inhibitors. Cells were washed and lysed as described (21,22), and lysates were subjected to immunoblot analysis.

Chemotaxis assay

PDGF-dependent chemotaxis was assayed utilizing a 48-well modified Boyden chemotaxis chamber (NeuroProbe Inc., Baltimore, Maryland) and collagen-coated polyvinylpyrrolidone-free polycarbonate filters (8-μm pore size) (Poretics Corp., Livermore, California) as described (21,22).

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DNA synthesis assay

DNA synthesis was measured by a 5-bromodeoxyuridine (BrdU)-incorporation assay, carried out according to the manufacturer's specifications (Roche, Mannheim, Germany) with an incubation time of 5 h as previously described (22).

Statistical analysis

All data are expressed as mean ± SEM. Statistical significance of differences was calculated using analysis of variance with post-hoc Tukey test or Student unpaired *t* test. $p < 0.05$ was considered statistically significant.

Results

The main goal of our study was to investigate whether selective blunting of PDGF-dependent VSMC proliferation and migration is sufficient to prevent neointima formation after vascular injury in vivo. To identify the crucial targets for neointima prevention, we performed in vitro studies utilizing chimeric CSF1R/βPDGFR mutants (ChiR) which contain the ligand-binding domain of the CSF1R (*c-fms*) and the cytoplasmic domain of the WT or mutated βPDGFR (17). The subtraction panel contains mutants in which the binding site for either one of the βPDGFR -associated signaling enzymes is mutated (Fig. 1A). The add-back panel is based on the F5 receptor and contains mutants that have the binding sites for one of the associated proteins restored (Fig. 1B). A kinase inactive mutant (R634) served as a negative control. The chimeric approach enabled us to selectively stimulate PDGF-dependent signaling cascades via ChiR mutants by M-CSF and to bypass endogenous βPDGFR in VSMCs. The ChiR mutant-expressing cell lines have been characterized previously (17).

Functionality of ChiR mutants in VSMCs

Because our approach required that VSMCs do not express functional CSF1R, we first compared their responsiveness to PDGF-BB and M-CSF. As expected, stimulation of non-transfected VSMCs with PDGF-BB led to tyrosine phosphorylation of the βPDGFR and to activation of downstream signaling molecules such as Erk1/2. In contrast, M-CSF did not mediate the tyrosine phosphorylation of the CSF1R and did not induce Erk phosphorylation in VSMCs (Fig. 2A), whereas it triggered these responses in human monocytes (not shown). M-CSF triggered the phosphorylation of downstream signaling molecules such as Akt, Erk, and p38, and VSMC migration and proliferation in ChiR-WT–expressing cells to a similar extent as PDGF-BB in non-transfected cells (Figs. 2B, 2C, and 2D). These data demonstrate that the system of ChiR mutants allows the systematic evaluation of βPDGFR signaling in VSMCs without the need for pharmacological inhibitors.

PI3-kinase and phospholipase Cγ mediate the βPDGFR's mitogenic signal

Stimulation of ChiR-WT expressing cells resulted in a concentration-dependent increase in BrdU uptake (Fig. 3A). Deletion of the binding sites for PI3K (F40/51) or PLCγ (F1021) almost completely suppressed DNA synthesis even at saturating ligand concentrations, whereas deletion of the Src binding site (F79/81) led to an enhanced mitogenic response. The latter is consistent with previous reports, identifying Src as a negative regulator of PDGFR signaling (21). ChiR mutants that only bind either PI3K (Y40/51) or PLCγ (Y1021) were able to partially mediate the ChiR-WT response (Fig. 3B). A potential caveat of utilizing receptor mutants is the possibility that in addition to the known binding partners of phosphotyrosines, other unknown signaling molecules may also interact with these sites. Therefore, all measurements were repeated in a second approach in PDGF-stimulated VSMCs in the presence of pharmacological inhibitors. Consistent with the findings obtained

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by the ChiR system, pharmacological inhibition of PI3K or PLCγ suppressed PDGF-BB mediated BrdU incorporation (Fig. 3C). Furthermore, inhibition of MEK1/2 also led to a decrease of VSMC proliferation, whereas suppression of p38 activity had no effect.

Multiple signaling enzymes are required for PDGF-dependent chemotaxis

Stimulation of ChiR-WT expressing VSMCs led to an increase in cell migration to approximately 4-fold the basal level (Fig. 4A). Mutation of the Src binding site (F79/81) almost completely abolished M-CSF–induced chemotaxis, and mutation of the binding sites for PI3K (F40/51) or PLCγ (F1021) significantly reduced M-CSF–dependent chemotaxis by 55% and 60%, respectively. Activation of either 1 signaling molecule alone (add-back panel) was not sufficient to rescue the chemotactic response (Fig. 4B). Consistent with the use of ChiR mutants, pharmacological inhibition of Src abolished PDGF-dependent chemotaxis in naive VSMCs, whereas inhibition of either PI3K or PLCγ led to a partial inhibition of this response (Fig. 4C). These data indicate that PI3K, PLCγ, and Src are required for PDGF-dependent chemotaxis in VSMCs.

PI3K and PLCγ differentially regulate the expression of cyclin D1 and p27^{kip1}

Growth factors regulate the cell cycle from the exit from G0 until late G1. Phosphorylation of the retinoblastoma protein (Rb) depends on G1 cyclin-dependent kinases (cdks), which are activated by G1 cyclins and inhibited by cdk inhibitors. The main growth factor–induced events in early G1 are the up-regulation of cyclin D1 and the down-regulation of the cdk inhibitor p27^{Kip1} (23). Stimulation of the endogenous βPDGFR with PDGF-BB and ChiR-WT with M-CSF led to a time-dependent increase of cyclin D1 expression and degradation of p27^{kip1}, resulting in efficient Rb phosphorylation at 24 h (Figs. 5A and 5B). As expected, the F5 receptor did not influence p27^{kip1} and cyclin D1 levels or mediate Rb phosphorylation (not shown). When the various ChiR mutants were compared for their ability to regulate cell cycle controlling molecules, disruption of PI3K binding to the ChiR (F40/51) abolished the induction of cyclin D1, whereas binding of PI3K alone (Y40/51) was sufficient to induce cyclin D1 expression (Figs. 5C and 5D). Conversely, disruption of all other signaling pathways including PLCγ (F1021) did not affect M-CSF–dependent cyclin D1 induction (Fig. 5C, and not shown). Interestingly, the F40/51 mutation diminished the receptor's capability to down-regulate p27^{kip1} to a much lesser extent than the F1021 mutation (Fig. 5C). Additionally, the Y1021 mutant but not the Y40/51 mutant was able to efficiently down-regulate p27^{kip1} (Fig. 5D). Pharmacological inhibition of PI3K in naive VSMC completely suppressed PDGF-dependent cyclin D1 induction, whereas inhibition of PLCγ had no influence (Fig. 5E). Conversely, inhibition of PLCγ abolished the down-regulation of p27^{kip1}, whereas inhibition of PI3K had little effect. Hence induction of cyclin D1 is primarily mediated by PI3K, whereas PLCγ primarily promotes the degradation of p27^{kip1}.

Disruption of PDGF-dependent PI3K and PLCγ activity by a combination mutant

Based on the preceding findings, we generated a combination mutant that lacks the binding sites for PI3K and PLCγ (F3 receptor; Fig. 6A) in an attempt to completely abolish PDGF-dependent proliferation and migration. The chimeric F3 mutant was stably expressed in VSMCs to a similar level than the ChiR-WT and did not associate with either PI3K or PLCγ upon stimulation with M-CSF (Figs. 6B and 6C). VSMCs transfected with the F3 mutant were unable to mediate a chemotactic or mitogenic signal upon M-CSF stimulation (Figs. 6D and 6E) and failed to efficiently induce cyclin D1 or down-regulate p27^{kip1} in response to M-CSF (Fig. 6F). Hence our in vitro studies identified PI3K and PLCγ as main mediators of PDGF-dependent proliferation and migration of VSMCs, and the F3 mutant appeared as the ideal tool to verify their importance in vivo.

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Disruption of PDGF-dependent PI3K and PLCγ signaling abolishes neointima formation after balloon injury in vivo

To evaluate the importance of PDGF-dependent PI3K and PLCγ signaling for neointima formation in vivo, we performed balloon injury in carotid arteries of mice that are homozygous for the murine βPDGFR F3 mutant. VSMCs derived from βPDGFR$^{F3/F3}$ mice neither elicited a mitogenic signal, nor were they able to migrate toward PDGF (Figs. 7A and 7B). Balloon injury was performed in the common carotid arteries of male βPDGFR$^{F3/F3}$ mice and littermate WT controls. Three weeks after balloon injury, WT mice displayed a robust neointima formation at the site of vascular injury, and this response was dramatically reduced in mice harboring the F3 mutation (Fig. 7C). Consequently, the intimal area and the intima-media ratio were significantly decreased in βPDGFR$^{F3/F3}$ mice, whereas the patent lumen area was increased (Table 1). Outward remodeling in the βPDGFR$^{F3/F3}$ mice was excluded, and there were no detectable differences in SMC differentiation markers and extracellular matrix (ECM) components (Fig. 7C, Online Fig. 1A). Consistently, activation of βPDGFR signaling in ChiR–expressing VSMCs did not affect the expression of differentiation markers (smoothelin, smooth muscle myosin heavy chain, alpha actin) or induce ECM components such as collagens I and Va (Online Figs. 1B and 2). The decreased intimal area in F3/F3 mice was paralleled by a decreased number of neointimal nuclei and proliferating VSMCs, whereas the area per nucleus (ECM index) was equal in WT and βPDGFR$^{F3/F3}$ mice (Table 1), indicating that decreased VSMC accumulation rather than diminished matrix deposition accounts for the reduced neointima formation.

Discussion

In this study, we systematically analyzed the contribution of the activated βPDGFR and its associated signaling molecules to cellular responses of VSMCs in vitro and neointima formation in vivo. The data presented herein are consistent with the schematic diagram outlined in Figure 8 and allow 2 important conclusions: 1) signaling by the activated βPDGFR (particularly through PI3K and PLCγ) is crucial for neointima formation after vascular injury; and 2) disruption of specific signaling pathways is sufficient to attenuate pathogenic processes such as neointima formation and constrictive vascular remodeling in vivo.

Although previous studies that applied pharmacological approaches or neutralizing antibodies have indicated that PDGF is an important contributor to neointima formation (12,14), some studies argued against a significant role of PDGF. For instance, the systemic administration of the tyrosine kinase inhibitor imatinib failed to reduce the recurrence of coronary in-stent restenosis after coronary interventions in humans (15). To directly prove the importance of βPDGFR-initiated signals for neointima formation and to identify the crucial signaling pathways, we took advantage of a targeted genetic modification. In mice that are homozygous for the F3 mutation, the βPDGFR is normally expressed but is unable to recruit and activate PI3K and PLCγ upon ligand binding (18). Based on the previous observations that deletion of either the βPDGFR or the PDGF-B gene resulted in embryonic lethality that was in part due to vascular defects, the fact that F3/F3 mice are viable and have no obvious phenotype appears rather surprising (18). However, chimeric analysis revealed that when F3-receptor– bearing cells competed with WT βPDGFR-bearing cells in the same animals, there was a strong selection against βPDGFR$^{F3/F3}$ cells in the layer of smooth muscle cells surrounding the vasculature. This finding, which indicates that the F3 mutant receptor does not transmit signals as efficiently as the WT receptor in vivo, prompted us to hypothesize that the F3 mutation may become relevant under pathological conditions. Indeed, when we performed balloon injury in the carotid arteries of βPDGFR$^{F3/F3}$ mice, the remodeling process at the site of vascular injury was profoundly reduced when compared with that of WT mice.

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Our results are in line with findings of other investigators showing that inhibition of βPDGFR signaling by the use of tyrosine kinase inhibitors or neutralizing antibodies prevented restenosis formation (12,14), as well as other consequences of vascular remodeling, such as atherogenesis and pulmonary arterial hypertension (24,25). The failure of oral imatinib to prevent the recurrence of in-stent restenosis in humans as shown by Zohlnhöfer et al. (15) does not discard the role of PDGF in this context, but may be explained by several reasons, including insufficient dosage, nonspecific inhibition of several tyrosine kinases instead of specific PDGF receptor inhibition, systemic instead of local application, and subtherapeutic tissue concentrations. Indeed, it has been suggested that imatinib administered orally may not reach sufficient serum concentrations (>10 μmol/l) to function as a potent inhibitor of PDGFR signaling in tissues (26). Our genetic approach circumvents the problem that a pharmacological agent may not reach sufficient tissue concentrations or may inhibit other unknown kinases and clearly showed that blunting of PDGF-dependent PI3K and PLCγ activity in vivo dramatically reduces neointima formation and constrictive arterial narrowing after vascular injury.

PDGF is known to exert mitogenic and migratory responses in VSMCs, both of which are thought to contribute to neointima formation. Previous studies have shown that neointimal VSMC accumulation in the first days after balloon injury occurs as a result of PDGF-dependent VSMC migration from the media to the intima (27). Although we did not particularly determine VSMC migration in our in vivo model, we show that the βPDGFR F3 mutation significantly reduced the number of proliferating VSMCs in the intima at 21 days after injury and that mutation of the PI3K and PLCγ binding sites abolished both PDGF-dependent VSMC proliferation and chemotaxis in vitro. Taken together, these data indicate that PDGF-dependent migration and proliferation are both important for neointima formation and that disruption of PDGF mediated PI3K and PLCγ signaling attenuates this process after balloon injury by affecting both responses.

The approach to investigate vascular remodeling in βPDGFR$^{F3/F3}$ mice was based on in vitro experiments utilizing chimeric CSF1R/βPDGFR mutants that allowed the systematic characterization of βPDGFR signaling in VSMCs. We found that multiple signaling enzymes including PI3K, PLCγ, and Src contribute to PDGF-dependent chemotaxis. Because the directed migration of cells requires a number of cellular events, it is likely that each of the signaling molecules contributes to PDGF-mediated chemotaxis in a different way. Previous studies have indicated that PI3K- and PLCγ-dependent pathways are involved in actin rearrangement, cytoskeletal changes, and lamellipodia formation (28–31), whereas Src activity may be crucial for the direction of cellular movement by mediating receptor tyrosine kinase internalization and cap formation in 1 region of the cell (32). In contrast to chemotaxis, βPDGFR-mediated cell cycle progression depends on 2 of the receptor-associated signaling molecules, PI3K and PLCγ. These results are consistent with a previous study in HepG2 cells (33). Although deletion of the binding sites for PI3K or PLCγ almost completely abrogated DNA synthesis, the restoration of either binding site in the F5 receptor partially rescued βPDGFR-mediated proliferation. This may be explained by the fact that in contrast to the add-back mutants, the subtraction mutants (F40/51 and F1021) both bind and activate negative regulators of PDGFR signaling such as RasGAP and SHP-2 (16). Rat VSMCs expressing the F3 mutant and murine VSMCs isolated from βPDGFR$^{F3/F3}$ mice were not able to mediate a mitogenic or chemotactic signal, indicating that the inability of the βPDGFR to associate with PI3K and PLCγ fully abrogated these responses. Our genetic approach bears one potential caveat: We cannot completely rule out that proteins other than the known binding partners of the mutated tyrosine residues also associate with these sites and thereby contribute to βPDGFR-mediated proliferation or migration. However, this seems very unlikely because pharmacological inhibition of PI3K or PLCγ also abrogated these cellular responses. In addition to mouse and rat VSMCs, we found that

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pharmacological inhibition of PI3K and PLCγ also attenuated PDGF-induced chemotaxis and proliferation of human coronary VSMCs (not shown), suggesting species independency.

Our further investigations focused on cyclin D1 and p27^{kip1}, which are critical for both cell cycle progression and chemotaxis (23,34,35). Although the role of PI3K for the regulation of cell cycle–controlling proteins has been addressed in multiple studies, the importance of PLCγ in this context was less clear. Our data indicate that PI3K and PLCγ regulate cell cycle progression at least in part in a non-redundant manner. PI3K was identified as the main regulator of βPDGFR-dependent cyclin D1 expression, whereas PLCγ appeared to be of minor importance for this event. This is consistent with other studies demonstrating that cyclin D1 is mainly regulated by PI3K in numerous systems (36–38). Surprisingly, our comparative analysis revealed that PLCγ is the main mediator of p27^{kip1} down-regulation. A recent report demonstrated that PDGF-BB–dependent degradation of p27^{Kip1} in VSMC is mediated by shortening the half-life of p27^{Kip1} mRNA via an Erk1/2-dependent pathway (39). Conventional protein kinase C isoforms that act downstream of PLCγ and contribute to p27^{Kip1} degradation are known to activate Erk1/2. Consistently, we found that inhibition of either Erk1/2 or protein kinase C almost completely abrogated PDGF-dependent down-regulation of p27^{Kip1} (not shown), suggesting a prominent role for these PLCγ effectors for the regulation of p27^{Kip1}. Nevertheless, p27^{kip1} seems to be differentially regulated in different cell types and through different growth factors, as PDGF-dependent degradation of p27^{kip1} in fibroblasts occurs independently from PI3K and is controlled by *c-myc*, whereas interleukin-2–induced repression of p27^{kip1} requires PI3K and Akt (40). Hence, growth factor–dependent degradation of p27^{kip1} may be targeted in a cell-type–specific manner. Although both pathways were found to be redundant to some extent, our comparative approach identified PI3K as the main regulator of PDGF-dependent induction of cyclin D1 in VSMCs, whereas PLCγ mainly controls the expression of p27^{kip1}.

Conclusions

In summary, our data indicate that PI3K and PLCγ mediate βPDGFR-dependent proliferation and migration of VSMCs and provide direct proof that these signaling pathways are essential for neointima formation after vascular injury in vivo. The identification of these signal relay mechanisms provides the basis for the development of novel therapeutic and preventive strategies that selectively target VSMCs without affecting endothelial cell function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank Andrius Kazlauskas and Alex Toker (Harvard Medical School) for generously supplying antibodies against the βPDGFR (97A), RasGAP (69.3), and p85; and Jessica Schnitker and Deborah Vieth for excellent technical assistance. This manuscript contains parts of a doctoral thesis by F.G.

Acknowledgments

This work was supported in part by the Deutsche Forschungs-gemeinschaft (Grants No. Ro 1306/2-1 and 2-2), the Center for Molecular Medicine Cologne (project A6), and the Marga und Walter Boll-Stiftung (Kerpen, Germany).

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APPENDIX

For accompanying data and supplemental figures, please see the online version of this article.

Abbreviations and Acronyms

BrdU	5-bromodeoxyuridine
cdk	cyclin-dependent kinase
ChiR	chimeric receptor
CSF1R	colony-stimulating factor-1 receptor
ECM	extracellular matrix
M-CSF	macrophage colony-stimulating factor
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol 3-kinase
PLCγ	phospholipase Cγ1
RasGAP	GTPase activating protein of ras
VSMC	vascular smooth muscle cell
WT	wild type

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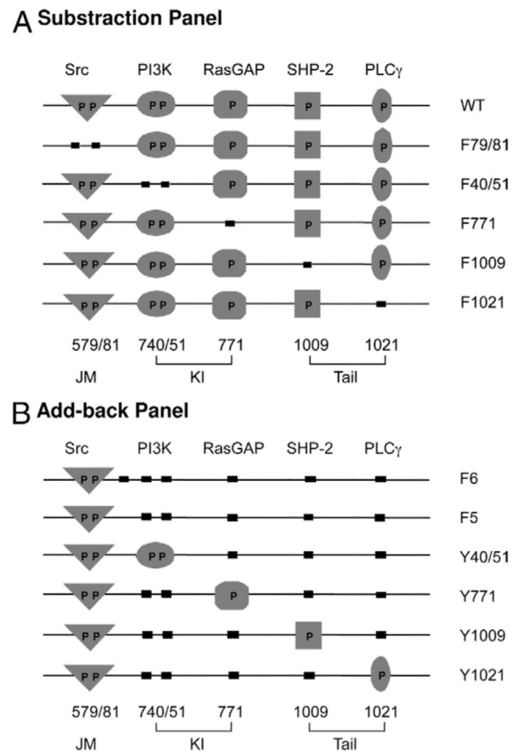

Figure 1. Schematic Diagram Illustrating the Cytoplasmic Domain of Chimeric Colony-Stimulating Factor 1 Receptor/Platelet-Derived Growth Factor Beta Receptor (ChiR) Mutants (17)

Tyrosine phosphorylation sites are represented as P, and Tyr-to-Phe substitutions are indicated as **black squares**. The signaling enzymes that associate with the receptor are shown at the **top** of the schemes. The nomenclature of the substraction panel **(A)** and add-back panel **(B)** of chimeric receptor (ChiR) mutants is indicated to the **right** of each receptor representation. In the subtraction panel, the names indicate which of the Tyr residues have been replaced with Phe (“F-mutants”), and in the add-back panel, the name of each mutant denotes which of the mutations in the F5 construct has been repaired (“Y-mutants”). JM = juxtamembrane; KI = kinase insert; PI3K = phosphatidylinositol 3-kinase; PLCγ = phospholipase Cγ1; RasGAP = GTPase activating protein of ras.

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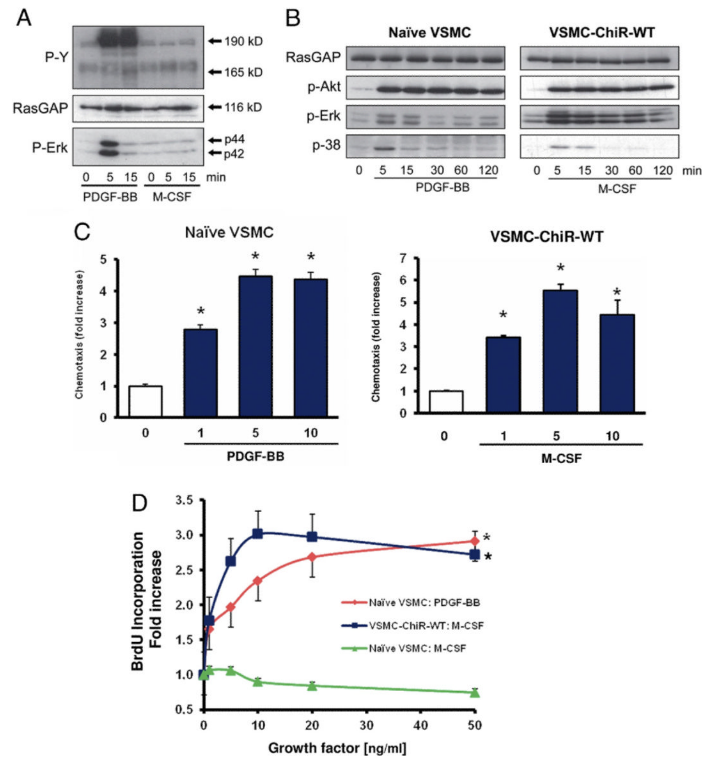

Figure 2. The M-CSF–Stimulated ChiR-WT Mimics PDGF-Induced Responses in VSMCs

(A) Responsiveness of naive vascular smooth muscle cells (VSMCs) to platelet-derived growth factor (PDGF)-BB (50 ng/ml) and macrophage colony-stimulating factor (M-CSF) (50 ng/ml). Shown are immunoblots probed with antibodies recognizing phospho-tyrosine (P-Y), GTPase activating protein of ras (RasGAP) (lysate control), and phospho-Erk. **(B)** Activation of downstream signaling molecules in PDGF-BB–stimulated naive VSMCs and M-CSF–stimulated chimeric receptor (ChiR) wild-type (WT)–expressing cells. **(C)** Comparison of the chemotactic response of naive VSMCs to PDGF-BB (10 ng/ml) and ChiR-WT–expressing cells to M-CSF (10 ng/ml). **(D)** Comparison of growth factor–dependent cell cycle progression in PDGF-BB– or M-CSF–stimulated naive or ChiR-WT–expressing VSMCs as indicated. Data represent means ± standard error of the mean from at least 3 independent experiments (*$p < 0.05$ vs. buffer).

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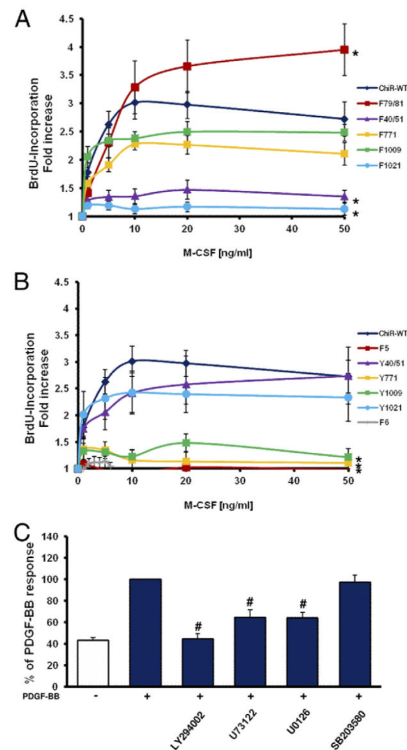

Figure 3. Role of Signal Relay Enzymes in PDGF Beta Receptor–Mediated DNA Synthesis
Vascular smooth muscle cells (VSMCs) expressing either the chimeric receptor (ChiR) wild-type (WT), the subtraction panel of ChiR mutants **(A)**, or the add-back panel of ChiR mutants **(B)** were arrested by serum deprivation and exposed to buffer or various concentrations of macrophage colony-stimulating factor (M-CSF). DNA synthesis was measured by 5-bromodeoxyuridine (BrdU) incorporation. Data are expressed as fold increase over buffer. **(C)** Effect of pharmacological inhibitors against phosphatidylinositol 3-kinase (LY294002), phospholipase Cγ1 (U73122), MEK (U0126), and p38 (SB203580) on platelet-derived growth factor (PDGF)-BB–dependent cell cycle progression in non-transfected VSMCs. Data are expressed as the percentage of PDGF-BB–stimulated cells. All data represent mean ± standard error of the mean from at least 3 independent experiments (*$p < 0.05$ vs. WT at 50 ng/ml M-CSF, #$p < 0.05$ vs. PDGF alone).

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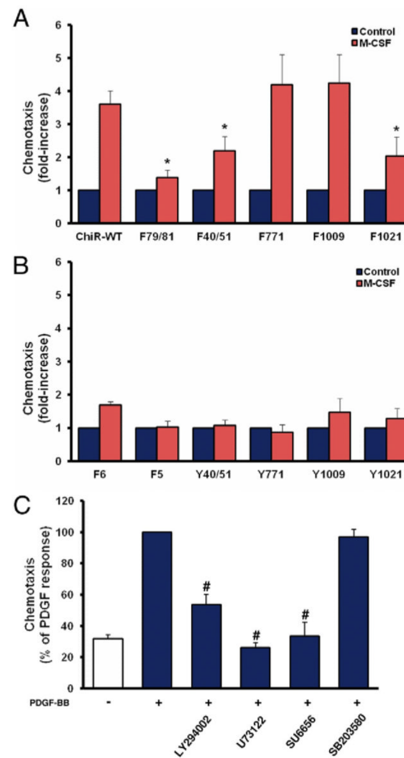

Figure 4. Role of Signal Relay Enzymes in PDGF Beta Receptor–Mediated Chemotaxis

(A) Subtraction panel of chimeric receptor (ChiR) mutants. **(B)** Add-back panel of ChiR mutants. **(C)** Naive vascular smooth muscle cells that have been stimulated with platelet-derived growth factor (PDGF)-BB in the presence of pharmacological inhibitors against phosphatidylinositol 3-kinase (LY294002), phospholipase Cγ1 (U73122), Src (SU6656), and p38 (SB203580). All data represent mean ± standard error of the mean from at least 3 independent experiments (*p < 0.05 vs. wild type; #p < 0.05 vs. PDGF alone).

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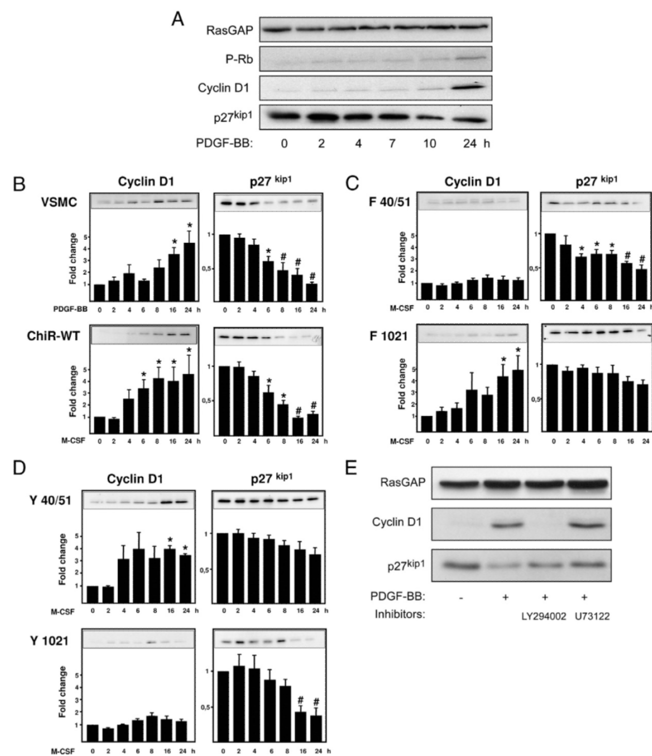

Figure 5. Regulation of Cell Cycle–Controlling Proteins Through PI3K and PLCγ

(A) Platelet-derived growth factor (PDGF)-BB causes induction of cyclin D1 and down-regulation of p27 ^{kip1} protein at 24 h, resulting in retinoblastoma protein phosphorylation (P-Rb). **(B)** The activated chimeric receptor wild type (ChiR-WT) mimics the PDGF-BB–induced response in naive vascular smooth muscle cells (VSMCs). **(C)** Regulation of cyclin D1 and p27 ^{kip1} by the ligand-induced F40/51 and F1021 mutants. **(D)** Regulation of cyclin D1 and p27 ^{kip1} by the ligand-induced Y40/51 and Y1021 mutants. **(E)** Naive VSMCs were treated with 50 ng/ml of PDGF-BB for 24 h in the presence of pharmacological inhibitors of phosphatidylinositol 3-kinase (PI3K; LY294002) or phospholipase Cγ1 (PLCγ; U73122). **(B to D)** Cyclin D1 and p27^{kip1} signals were scanned and quantified by densitometry, and the values were normalized for GTPase activating protein of ras (RasGAP) levels (lysate control). Data represent mean ± standard error of the mean from 3 independent experiments (*p < 0.05, #p < 0.01 vs. buffer).

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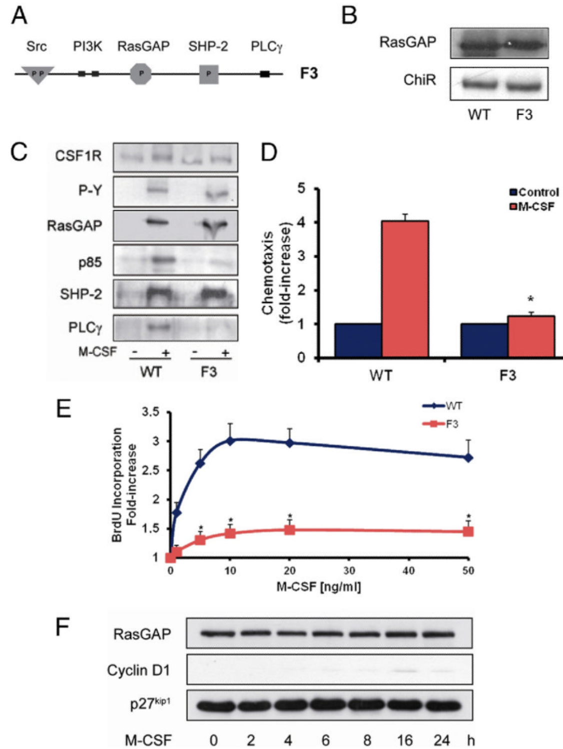

Figure 6. Blunting of Mitogenic and Chemotactic Signaling by a Combination Mutant That Lacks the PI3K and PLCγ Binding Sites (F3 Receptor)

(A) Schematic diagram illustrating the cytoplasmic domain of the chimeric receptor (ChiR)-F3 mutant harboring Tyr-to-Phe substitutions at tyrosine residues 740/51 and 1021. **(B)** Expression levels of ChiR-F3 in comparison with ChiR-wild type (WT)–expressing cells. **(C)** Binding characteristics of platelet-derived growth factor beta receptor–associated signaling molecules in quiescent and macrophage colony-stimulating factor (M-CSF)–stimulated ChiR-WT and ChiR-F3 expressing cells. **(D, E)** M-CSF–dependent chemotaxis and cell cycle progression in WT and ChiR-F3 expressing vascular smooth muscle cells (VSMCs). **(F)** Effect of M-CSF on cyclin D1 and $p27^{kip1}$ levels in ChiR-F3 expressing VSMCs. Data in **D** and **E** represent mean ± SEM from 3 independent experiments (*$p < 0.05$ vs. WT). BrdU = 5-bromodeoxyuridine; CSF1R = colony-stimulating factor-1 receptor; other abbreviations as in Figure 1.

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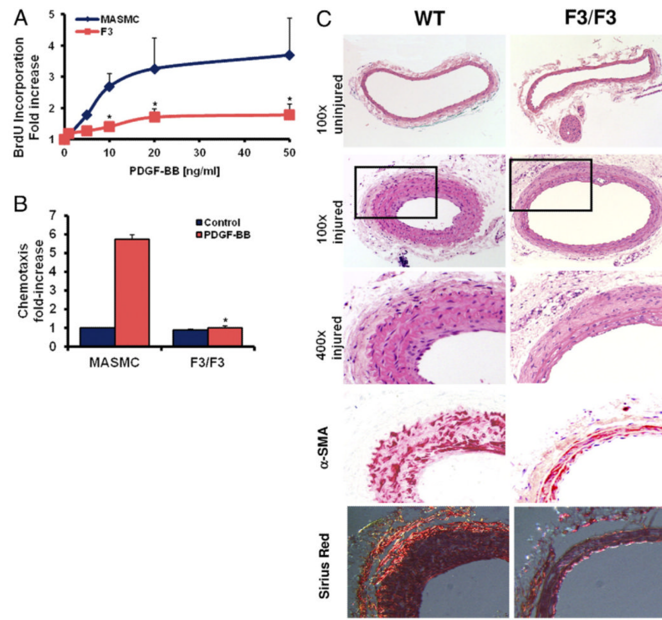

Figure 7. Disruption of PDGF-Dependent PI3K and PLCγ Signaling Attenuates Neointima Formation In Vivo

(A, B) Platelet-derived growth factor (PDGF)–dependent cell cycle progression and chemotaxis in vascular smooth muscle cells isolated from wild type (WT) or PDGF beta receptor (βPDGFR)-F3/F3 mice (*$p < 0.05$ vs. WT-mouse aortic smooth muscle cells [MASMC]). **(C)** Representative histological cross-sections (hematoxylin eosin staining; 100×; inset: 400×; alpha-smooth muscle actin and Sirius red staining) of uninjured and injured common carotid arteries from male βPDGFR$^{F3/F3}$ mice and littermate WT controls 3 weeks after balloon injury.

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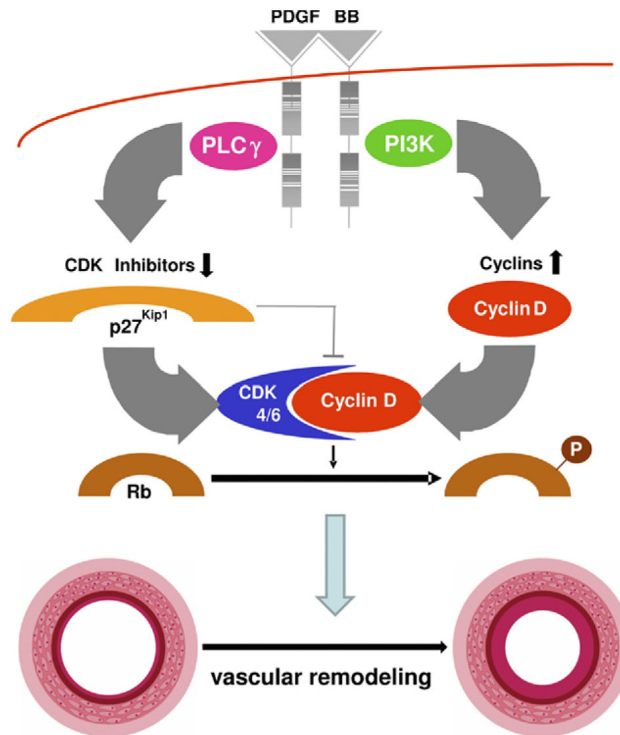

Figure 8. Schematic Diagram Illustrating the Differential Roles of PDGF-Dependent PI3K and PLCγ Activity for Cellular Responses and Neointima Formation In Vivo

Upon ligand binding, the platelet-derived growth factor (PDRF) beta receptor recruits multiple signaling molecules, including phosphatidylinositol 3-kinase (PI3K) and phospholipase Cγ1 (PLCγ). Although PI3K is required for efficient up-regulation of cyclin D1, PLCγ is mainly responsible for down-regulation of $p27^{kip1}$. Both events are required for retinoblastoma protein (Rb) phosphorylation, which is crucial for cellular events such as cell cycle progression and chemotaxis. At least in mice, these events appear to be crucial for neointima formation after balloon injury. CDK = cyclin-dependent kinase.

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Table 1

Morphometric Analyses of Carotid Arteries From WT or Platelet-Derived Growth Factor Beta Receptor F3/F3 Mice 21 Days After Balloon Injury

	Intimal Area (μm^2)	Medial Area (μm^2)	Lumen Area (μm^2)	Intima-Media Ratio
WT (n = 10)	19,276 ± 5,409	30,071 ± 2,158	44,982 ± 9,130	0.64 ± 0.19
F3/F3 (n = 11)	6,309 ± 978 *	28,887 ± 3,527	79,780 ± 8,689 *	0.23 ± 0.03 *
	Intimal Nuclei	**ECM Index (μm^2/cell)**		**PCNA Positive Cells (%)**
WT (n = 8)	88.58 ± 29.66	359.8 ± 78.4		16.79 ± 2.44
F3/F3 (n = 7)	28.19 ± 10.43	427.6 ± 68.2		5.48 ± 0.83 *

Data are represented as mean ± SEM.

* $p < 0.05$ versus WT.

ECM = extracellular matrix; PCNA = proliferating cell nuclear antigen; WT = wild-type.