Endothelial and smooth muscle cell-derived neuropilin-like protein regulates platelet-derived growth factor signaling in human vascular smooth muscle cells by modulating receptor ubiquitination

Received for publication, July 28, 2009. Published, JBC Papers in Press, August 19, 2009, DOI 10.1074/jbc.M109.049684

Xiaojia Guo1,2, Lei Nie1,2, Leila Esmailzadeh1,3, Jiasheng Zhang1,3, Jeffrey R. Bender1,3, and Mehran M. Sadeghi1,2

From the 1Cardiovascular Molecular Imaging Laboratory, the 1Raymond and Beverly Sackler Foundation Cardiovascular Laboratory, Section of Cardiovascular Medicine, and the 3Interdepartmental Program in Vascular Biology and Therapeutics, Yale University School of Medicine, New Haven, Connecticut 06520 and the 2Veterans Affairs Connecticut Healthcare System, West Haven, Connecticut 06516

Endothelial and smooth muscle cell-derived neuropilin-like protein (ESDN) is up-regulated in the neointima of remodeling arteries and modulates vascular smooth muscle cell (VSMC) growth. Platelet-derived growth factor (PDGF) is the prototypic growth factor for VSMCs and plays a key role in vascular remodeling. Here, we sought to further define ESDN function in primary human VSMCs. ESDN down-regulation by RNA interference significantly enhanced PDGF-induced VSMC DNA synthesis and migration. This was associated with increased ERK1/2, Src, and PDGF receptor (PDGFR) phosphorylation, without altering total PDGFR expression levels. In binding assays, ESDN down-regulation significantly increased 125I-PDGF maximum binding (Bmax) to PDGF receptors on VSMCs without altering the binding constant (Kd), raising the possibility that ESDN regulates PDGFR processing. ESDN down-regulation significantly reduced ligand-induced PDGFR ubiquitination. This was associated with a significant reduction in the expression level of c-Cbl, an E3 ubiquitin ligase that ubiquitinates PDGFR. Thus, ESDN modulates PDGF signaling in VSMCs via regulation of PDGFR surface levels. The ESDN effect is mediated, at least in part, through effects on PDGFR ubiquitination. ESDN may serve as a target for regulating PDGFR signaling in VSMCs.

Vascular injury initiates a cascade of events that ultimately leads to vascular remodeling and often intimal hyperplasia. Vascular smooth muscle cell (VSMC) proliferation and migration are key cellular events in this process. Platelet-derived growth factor (PDGF)-BB is released by platelets, endothelial cells, VSMCs, and inflammatory cells at the sites of vascular injury and is a particularly potent regulator of VSMC proliferation and migration. PDGF binding to PDGF receptor (PDGFR)β in VSMCs leads to receptor dimerization, autophosphorylation, and activation of downstream signaling pathways, including MAPK. The ligand-bound receptor is internalized through the endocytic pathway and may either recycle to the membrane or undergo ubiquitination and lysosomal degradation. A number of endogenous stimulatory and inhibitory regulators, including the E3 ubiquitin ligase, c-Cbl, tightly regulate the mitogenic stimulus by modulating the duration and intensity of the signal.

We have identified endothelial and smooth muscle cell-derived neuropilin-like protein (ESDN, also called CLCPI or DCBLD2) as a marker and regulator of cell proliferation in vascular remodeling. ESDN is a transmembrane protein with a domain structure similar to neuropilins. ESDN can be induced by PDGF-BB and serum and is highly expressed in the neointima of injured rat, mouse, and human arteries. ESDN expression parallels cell proliferation in the vessel wall in vivo. Furthermore, ESDN is up-regulated in proliferating VSMCs, and ESDN overexpression inhibits VSMC growth. Here, we expand the scope of our previous studies to demonstrate that ESDN regulates PDGF-induced VSMC migration and inhibits PDGF signaling in VSMCs. We further establish that this effect is mediated, at least in part, through changes in the surface expression of PDGF receptors. Finally, our study indicates that ESDN mediates PDGFRβ ubiquitination by regulating c-Cbl gene expression.

EXPERIMENTAL PROCEDURES

Materials—Reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless stated otherwise. Human recombinant PDGF-BB was purchased from R&D Systems, Inc. (Minneapolis, MN). [3H]thymidine was purchased from Amersham Biosciences Pharmacia (GE Healthcare) and 125I-PDGF was from PerkinElmer Life Sciences. The following antibodies were used: anti-ESDN (Sigma), anti-PDGFRβ, anti-phospho PDGFRβ (Tyr1021), and anti-ubiquitin monoclonal antibody (P4D1) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-ERK1/2, anti-phospho ERK1/2 (Thr202/Tyr204), anti-Src, anti-phospho...
Src (Tyr\(^{116}\)), anti-c-Cbl, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology, Beverly, MA); and anti-hemagglutinin (Roche Applied Science). M199 and Opti-MEM I were obtained from Invitrogen. Horseradish peroxidase-labeled streptavidin was from Millipore Corp. (Temecula, California). Protein A/G agarose beads were from Santa Cruz Biotechnology. Complete protease inhibitor mixture, EDTA-free, was purchased from Roche Applied Science.

Cells—Primary human VSMCs were isolated as described (4) from human aortas, obtained under protocols approved by the Yale University Human Investigation Committee, and grown in M199 with 20% fetal bovine serum. Only VSMCs within the first four passages were utilized. Each experiment was repeated with VSMCs from at least three different donors.

RNA Interference—Three 25-bp duplex RNA that specifically target different regions of the human ESDN mRNA (Acc NM_080927) were designed and synthesized by Invitrogen: ESDN2245: 5′-GGA AUU GUU GGU ACA CUU CAU CAA A; ESDN769: 5′-GGA AUU GGA GUC AGC AGA ACU GAA A; ESDN1081: 5′-GUG CAU GCA GGA GUA GUG UCA A. VSMCs were seeded in 60-mm plates. After 12–24 h incubation in antibiotic-free M199 containing 20% fetal bovine serum at 37 °C and 5% CO\(_2\), cells were transfected with either 100 nM ESDN or a universal negative control (Invitrogen) small interfering RNA (siRNA) in Opti-MEM I using FuGENE 6 transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions.

Quantitative Reverse Transcription-PCR (RT-PCR)—Relative transcript expression levels of various genes were assessed by real time RT-PCR. Total RNA extraction, cDNA synthesis, and PCR conditions were performed as described previously (4). PCR primers used in these studies were synthesized by Yale University Keck Facility, and their sequences are listed in supplemental Table 1. For quantitative RT-PCR analysis, each template was tested in triplicate. The abundance of each gene was determined relative to GAPDH.

\( ^{3}\text{H} \)Thymidine Incorporation Assay—DNA synthesis was quantitated by measuring \( ^{3}\text{H} \)thymidine incorporation. VSMCs transfected with ESDN or green fluorescent protein retrovirus (4) and VSMCs transfected with either ESDN or control siRNA for 48 h seeded on 24-well culture plates at a density of 1 × 10\(^4\) cells/well were serum-starved overnight and treated with PDGF (25 ng/ml) or control buffer in eight replicate wells for 24 h. During the last 6 h, the cells were incubated with 0.5 \( \mu l \)/well of \( ^{3}\text{H} \)thymidine (0.0185 MBq; GE Healthcare). After rinsing, cells were fixed in ice-cold methanol, and DNA was precipitated by 5% trichloroacetic acid and recovered with NaOH (0.3 \( \text{n} \)) at room temperature. Aliquots were assayed for \( ^{3}\text{H} \)thymidine incorporation by liquid scintillation counting (Beckman Coulter, Inc. Fullerton, CA). Counts were normalized to the control sample and expressed as relative \( ^{3}\text{H} \)thymidine incorporation.

Migration Assay—Forty-eight h after siRNA transfection, confluent VSMC monolayer cultures were placed in serum-free medium. Twenty-four h later, cells were scratched with a scraper to create a wound. After wounding, the medium was changed to serum-free medium with or without PDGF-BB (25 ng/ml). Random fields of view were photographed with phase-contrast microscope (Nikon Instruments, Inc., Melville, NY) at baseline and after 24 h. The distance between the leading edge of migrating cells and the wound edge was measured and averaged on 10 random photos for each experimental sample using NIH ImageJ software. The cells that migrated out from wound edge were counted from 10 random photos as well.

Immunoblotting—Forty-eight h following transfection with siRNAs, VSMCs were serum-starved for 24 h, followed by stimulation with PDGF (25 ng/ml) or control buffer for the indicated time. Cells were washed twice with chilled phosphate-buffered saline (PBS) and lysed in ice-cold lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, and complete protease inhibitor (Roche Diagnostics) and phosphatase inhibitor (Sigma) cocktails). Cell homogenates were centrifuged (13,000 \( \times \) g, 15 min, 4 °C), supernatants were collected, and protein concentrations were determined (DC protein assay reagent, Bio-Rad). Equal amounts of protein were resolved by SDS-PAGE and transferred electrophoretically to an Immunoblot polyvinylidene difluoride membrane (Bio-Rad). The membranes were probed with primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies (Zymed Laboratories Inc., Carlsbad, CA) and developed using a chemiluminescence detection system (PerkinElmer Life Sciences). GAPDH was used as loading control. Films were scanned and quantitated using Kodak 1D 3.5 software.

Immunoprecipitation—Cell lysates (containing 500 \( \mu g \) of protein) were incubated with 20 \( \mu l \) of protein A-agarose beads for 1 h at 4 °C, after which the beads were removed by centrifugation to clear the lysate. Precleared lysates were incubated with the respective antibodies for 1 h at 4 °C and subsequently incubated with 15 \( \mu l \) of protein A-agarose beads ans/or protein G-agarose beads for 1 h at 4 °C, followed by washing with lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, and complete proteinase inhibitor; Roche Applied Science). Immunocomplexes were resuspended in 15 \( \mu l \) of SDS-PAGE sample buffer.

PDGF Binding Assay—VSMCs transfected with ESDN or control siRNA for 48 h were plated in complete growth medium in 96-well plates at 3 × 10\(^3\) cells/well. Twenty-four h after seeding, cells were rinsed with PBS and incubated in M199 with 0.5% fetal bovine serum containing increasing concentrations of \(^{125}\text{I} \)-PDGF-BB in quadruplicates at 4 °C. After 2 h, the cells were washed with ice-cold buffer and solubilized in RIPA buffer. Radioactivity in an aliquot of solubilized cells was measured by a gamma well counter (Cobra, Auto-Gamma, Packard Instrument Company). Cell numbers in parallel wells were determined after gentle trypsinization and used to normalize the results. Nonspecific binding was determined using 200-fold excess unlabeled PDGF-BB. Specific binding was calculated by subtracting nonspecific binding from total binding. The equilibrium binding constant (\( K_d \)) and maximum specific binding (\( B_{max} \)) were determined by non-linear regression (one-site specific binding) using GraphPad Prism software (La Jolla, CA).

Surface Labeling—To quantify surface PDGF\(\beta\), cell surface proteins were covalently labeled using a membrane-impermeant biotinylation reagent (NHS-SS-biotin; ThermoScien
tífic, Rockford, IL). Forty-eight h following transfection with
Esdn and Regulation of PDGF Signaling in VSMCs

RESULTS

ESDN Impairs PDGF-induced VSMC Proliferation—VSMC proliferation and migration, key cellular events in neointimal hyperplasia, are potently induced by PDGF. Therefore, we examined the effect of ESDN on these PDGF-induced cellular events. We have previously reported that ESDN overexpression inhibits VSMC growth, whereas ESDN knockdown shows an opposite effect (4). To establish whether the observed effect of ESDN on VSMC growth is, at least in part, through regulation of cell proliferation, we assessed the effect of ESDN knockdown on VSMC DNA synthesis. Three ESDN siRNA duplexes targeting distinct regions of the ESDN mRNA (769, 1081, 2245; the numbers refer to the oligonucleotide position within ESDN mRNA) were tested for their ability to reduce ESDN expression in cultured VSMCs. An siRNA (2245) targeting the mRNA sequence, GGAUUGUUGUACACUUCAA, was found to exert the greatest down-regulation of ESDN expression as evaluated by quantitative RT-PCR (to ~40%, Fig. 1A and also supplemental Fig. 1) without altering the stress responsive gene OAS1 (supplemental Fig. 1) in both control and PDGF-treated cells (supplemental Fig. 2). The inhibitory effect of this siRNA on the VSMC ESDN protein level was confirmed by Western blotting (Fig. 1B).

Therefore, we used this siRNA for the following experiments unless indicated otherwise. ESDN knockdown did not affect [3]H]thymidine incorporation (relative [3]H]thymidine incorporation 1 versus 0.95 ± 0.04, respectively, for control and ESDN siRNAs, p > 0.05) in unstimulated cells. However, PDGF-induced [3]H] incorporation was significantly higher in ESDN knockdown cells (relative [3]H]thymidine incorporation 14.8 ± 0.5 versus 22.5 ± 0.2, respectively, for control and ESDN siRNAs, p < 0.001) (Fig. 1C). To confirm the observation from siRNA down-regulation that ESDN inhibits PDGF-induced VSMC proliferation, we transduced primary human VSMCs with a retroviral vector encoding ESDN cDNA (4). Transduction efficiency assessed by flow cytometry using a control green fluorescent protein-expressing vector was estimated to be around ~50%. ESDN overexpression modestly attenuated PDGF-induced [3]H] incorporation (data not shown). Collectively, these results demonstrate the inhibitory role of ESDN on PDGF-induced VSMC proliferation.

ESDN Attenuates PDGF-induced VSMC Migration—Next, we addressed the effect of ESDN down-regulation on VSMC migration using a scratch wound assay. In the absence of growth factor, there was minimal cell migration over a period of 24 h (data not shown). In the presence of PDGF (25 ng/ml), siRNA-mediated ESDN inhibition significantly enhanced PDGF-induced VSMC migration as compared with cells transfected with the control siRNA (migration distance: 650 ± 20 and 920 ± 50 µm; and migrating cells per microscopic field: 33 ± 1 and 42 ± 1, respectively, for the control and ESDN siRNAs, p < 0.0001) (Fig. 2, A–E). Interestingly, PDGF-induced VSMC migration was proportional to the degree of ESDN reduction (supplemental Fig. 3). These results indicate that similar to its effects on VSMC proliferation, ESDN regulates PDGF-induced VSMC migration.

ESDN Regulates PDGF Signaling—PDGF-induced VSMC differentiation, proliferation, and migration is dependent on MAPK activation and the resultant Elk-1 phosphorylation and transcriptional activation of many growth factor-inducible genes (for review, see Ref. 7). To elucidate the mechanism of the ESDN effect on PDGF-induced VSMC proliferation and migration, we examined the effect of ESDN down-regulation on MAPK ERK1/2 phosphorylation by Western blotting. Transfection of VSMCs with ESDN siRNA markedly enhanced PDGF-induced ERK1/2 phosphorylation as compared with the
control siRNA (Fig. 3A and supplemental Fig. 4). In addition to ERK1/2 signaling, Src-mediated signaling cascade also plays a role in VSMC migration and mitogenic responses (8–10). Therefore, we assessed the effect of ESDN down-regulation on Src phosphorylation and demonstrated that ESDN inhibition by RNA interference enhances PDGF-induced Src phosphorylation (Fig. 3B). Together, these data indicate that ESDN modulates activation of several PDGF-related signaling pathways.

**PDGFR** is the major receptor for PDGF-BB in VSMCs, and its phosphorylation mediates PDGF-induced ERK1/2 and Src phosphorylation (11). As such, PDGFRβ stands out as a candidate for the observed effect of ESDN in regulating PDGF signaling in VSMCs. To explore a potential effect of ESDN on PDGFRβ, we assessed the effect of ESDN down-regulation on PDGFRβ expression and phosphorylation in VSMCs. ESDN down-regulation did not affect PDGFRβ mRNA level (data not shown), and its effect on its protein expression was minimal (Fig. 3C). However, it markedly enhanced PDGF-induced PDGFRβ phosphorylation in VSMCs (Fig. 3C).

**ESDN Alters the Number of PDGF Binding Sites on VSMCs**—The observed effect of ESDN down-regulation on PDGFRβ phosphorylation may be explained by a potential effect on VSMC surface receptor numbers or affinity for the ligand. Therefore, we evaluated the effect of ESDN on PDGF binding to its receptors on VSMCs using 125I-labeled PDGF-BB. VSMCs transfected with ESDN siRNA displayed significantly higher numbers of PDGF-BB binding sites as compared with control
ESDN and Regulation of PDGF Signaling in VSMCs

**FIGURE 4. Effect of ESDN down-regulation on saturation binding of \(^{125}\text{I}\)-PDGF to VSMCs.** Cells were transfected with either the control (■) or ESDN siRNA (▲) for 48 h, followed by 24 h of serum starvation. Cells were washed with ice-cold PBS and incubated with indicated concentrations of \(^{125}\text{I}\)-PDGF for 2 h at \(4^\circ\)C. After extensive washing, cells were lysed, and radioactivity was measured by \(\gamma\)-well counting. A, saturation profiles. Each data point represents the mean ± S.E. of quadruplicates. B, Scatchard plot obtained by transformation of saturation binding data. ESDN siRNA significantly increased \(B_{\text{max}}\) without altering \(K_d\). Similar results were obtained with VSMCs from three different donors.

**FIGURE 5. Effect of ESDN on PDGF-induced PDGFR\(\beta\) ubiquitination.** VSMCs were transfected with control or ESDN siRNAs for 2 days, followed by 24 h serum starvation, and then incubated with or without PDGF (25 ng/ml) for 0–60 min. Cell lysates (containing 0.5 mg of protein) were immunoprecipitated with an anti-PDGFR\(\beta\) antibody, followed by immunoblotting with an anti-ubiquitin (Ub) antibody (upper panel) or an anti-PDGFR\(\beta\) antibody (lower panel). The quantification represents means ± S.E. of three independent experiments. Level of ubiquitin (ubiquitylated PDGFR\(\beta\)) was normalized to total PDGFR\(\beta\) immunoprecipitated. *, \(p < 0.05\).

**FIGURE 6. Effect of ESDN on Cbl expression.** A, representative Western blot and quantification of relative protein levels of c-Cbl and ESDN in VSMCs transfected with either control or ESDN siRNA for 2 days, followed by 24 h serum starvation, and then incubated with or without PDGF (25 ng/ml) for 0–60 min. GAPDH was used as a control for protein loading. B, quantitative RT-PCR assessment of GAPDH-normalized c-Cbl mRNA expression. Total RNA was extracted from VSMCs transfected with either control or ESDN siRNA, reverse transcribed, and used for PCR amplification. The quantification represents means ± S.E. from three independent experiments. *, \(p < 0.05\).

**ESDN and Regulation of PDGF Signaling in VSMCs**

|                | Control siRNA | ESDN siRNA | \(p\) value |
|----------------|--------------|------------|-------------|
| **B\(_{\text{max}}\)** (fmol/10\(^6\) cells) | 492 ± 5      | 773 ± 9    | <0.0001     |
| **K\(_d\)** (pM)       | 656 ± 47     | 849 ± 61   | 0.1515      |

**ESDN Affects Ligand-induced Ubiquitination of PDGFR\(\beta\)—** Polyubiquitination of PDGFR\(\beta\) is a necessary step for receptor degradation in response to PDGF stimulation and plays a negative regulatory role on PDGFR\(\beta\) signaling (12, 13). This raises the possibility that PDGFR\(\beta\) ubiquitination plays a role in the observed ESDN effect on PDGFR\(\beta\) signaling. Therefore, we compared the levels of ubiquitinated PDGFR\(\beta\) after PDGF-BB stimulation in VSMCs transfected with ESDN or control siRNA. Ubiquitinated PDGFR\(\beta\) was detected by immunoblotting of immunoprecipitated PDGFR\(\beta\) with an anti-ubiquitin antibody. As expected, PDGF-stimulation led to PDGFR\(\beta\) ubiquitination and concomitant reduction in PDGFR\(\beta\) levels (Fig. 5A). Furthermore, the ratio of ubiquitinated to total PDGFR\(\beta\) was significantly lower in ESDN down-regulated, as compared with control cells (Fig. 5B). As such, PDGFR\(\beta\) ubiquitination, a negative regulator of PDGFR\(\beta\) signaling, is reduced by ESDN down-regulation, resulting in increased PDGFR\(\beta\) signaling.

**ESDN Regulates c-Cbl Expression—** c-Cbl is a cytoplasmic ubiquitin ligase that has been implicated as a negative regulator of PDGFR\(\beta\) signaling (3). To address the mechanism of the ESDN effect on PDGFR\(\beta\) ubiquitination, we investigated whether ESDN down-regulation affects VSMC c-Cbl protein level by Western blotting. In VSMCs transfected with ESDN siRNA c-Cbl was significantly reduced in conjunction with the reduction in ESDN protein level (Fig. 6A).

Finally, we sought to determine whether ESDN modulates c-Cbl protein level by regulating its gene expression. Transfecting primary human VSMCs with ESDN siRNA significantly reduced c-Cbl mRNA level in parallel with the expected reduction in ESDN mRNA level (to 61% ± 0.02% of the level observed in cells transfected with control siRNA) (Fig. 6B). ESDN siRNA transfection did not affect the expression of a stress responsive gene, OAS1, a cytoskeletal protein, \(\beta\)-actin, and a growth factor receptor, vascular endothelial growth factor receptor-1, in
ESDN and Regulation of PDGF Signaling in VSMCs

VSMCs (supplemental Fig. 1), indicating that the observed inhibitory effect on c-Cbl mRNA level is very specific.

DISCUSSION

ESDN was initially cloned from human coronary arterial (5) and highly metastatic lung cancer (6) cells. We previously demonstrated that in animal models of immune or mechanical injury-induced vascular remodeling, ESDN expression was temporally and spatially associated with cell proliferation, thus providing a potential regulatory mechanism for the response to injury (4). In vitro, ESDN overexpression suppressed and its down-regulation enhanced VSMC growth. Here, we extend the scope of those findings to demonstrate that ESDN down-regulation potentiates PDGF-induced VSMC DNA synthesis and migration, indicating that ESDN functions as a suppressor of PDGF signaling in VSMCs. Despite the relatively modest changes in ESDN mRNA and protein levels achieved by RNA interference in primary human VSMCs, the modulatory effects of ESDN on PDGF-induced migration and growth were consistently detectable. It is expected that more pronounced changes in the ESDN level (expected e.g. in the ESDN knock-out mouse) would lead to more striking effects on VSMC biology. These observations led us to further explore a potential regulatory effect of ESDN on PDGF signaling in VSMCs.

PDGF was identified more than 30 years ago as a serum growth factor for VSMCs and fibroblasts. The PDGF family consists of homo- or heterodimers of four distinct polypeptide chains, namely PDGF-A, -B, -C, or -D, which interact with two distinct receptors (PDGFRα and β). PDGF-BB, produced by endothelial cells, megakaryocytes, and neurons, is the prototypic growth factor for VSMCs. It is unique in its ability to induce profound suppression of VSMC differentiation marker genes through PDGFRβ signaling (1). The role of PDGF in triggering VSMC proliferation and migration is supported by in vitro as well as in vivo evidence. As such, PDGF-BB antibodies can inhibit VSMC proliferation (14) and migration (15) after carotid injury in the rat. Existing data suggest that these effects are mediated by PDGFRβ, but not PDGFRα, signaling as the latter cannot be activated by PDGF-BB (7). PDGF binding to PDGFRβ leads to receptor dimerization, autophosphorylation, and recruitment of Src homology 2 (SH2) domain-containing signaling molecules (16). This in turn leads to MAPK (ERK1/2, p38 MAPK, and stress-activated protein kinase/c-Jun N-terminal kinase) activation (17). The resultant ERK1/2-induced Elk-1 phosphorylation is a key event in PDGF-induced VSMC differentiation, proliferation, and migration (1, 18). As expected from its observed effects on VSMC proliferation and migration, ESDN down-regulation potentiated PDGF-induced ERK1/2, Src, and PDGFRβ phosphorylation, demonstrating that ESDN regulation of VSMC biology is, at least in part, linked to its effect on PDGFRβ signaling.

Upon binding to PDGF, PDGFRβ is phosphorylated, ubiquitylated, and internalized and undergoes lysosomal degradation or recycling to the cell membrane (19, 20). The E3 ubiquitin ligase, c-Cbl, is activated after PDGF stimulation and associates with phosphorylated PDGFRβ (21, 22). This in turn leads to PDGFRβ ubiquitination, internalization via the clathrin-mediated pathway, and lysosomal degradation negatively regulating PDGFRβ signaling and cell proliferation (21). In addition to ubiquitin ligase, SHP-2 tyrosine phosphatase (23) and T-cell protein tyrosine phosphatase (24) bind to, dephosphorylate, and negatively regulate PDGFRβ signaling. ESDN down-regulation increased the number of PDGF binding sites without significant changes in total receptor level, raising the possibility that it may alter receptor ubiquitination. It is known that Cbl-mediated enhanced lysosomal degradation limits the biological half-life of the only small but important active pool of receptor tyrosine kinases. This is thought to be an attenuation mechanism, regulating the global impact of receptor tyrosine kinase activation within a cell. It is not surprising that ubiquitination of the active PDGFRβ pool does not significantly affect total receptor levels. Indeed, we observed a clear decrease in PDGF-induced PDGFRα ubiquitination in ESDN down-regulated cells. This was associated with a reduction in c-Cbl protein and mRNA levels, providing a potential mechanism for the significantly reduced PDGF-induced PDGFRβ ubiquitination in ESDN down-regulated VSMCs. Of note, c-Cbl overexpression enhances PDGFRβ ubiquitination and subsequent degradation in NIH3T3 fibroblasts (21).

The data presented here support our speculation that ESDN up-regulation participates in controlling VSMC proliferation and migration in injury-induced vascular remodeling, potentially by reducing the response to growth factors in a negative feedback loop (4). Other regulators of VSMC proliferation and migration and neointima formation have been described. Similar to ESDN, fibulin-5 is minimally expressed in normal arteries, is up-regulated in conjunction with neointima formation in remodeling arteries, and negatively regulates VSMC proliferation and migration (25). Integrins, such as αvβ3 (26) and α7β1 (27, 28), are induced and modulate VSMC proliferation and migration in vascular remodeling at multiple levels, including potentiation of ligand-dependent and -independent growth factor receptor signaling (29) through matrix engagement. Although many studies have identified c-Cbl and other Cbl-family proteins as key regulators of receptor tyrosine kinase and other signaling pathways (for review, see (30)), to our knowledge, this is the first report of regulating receptor signaling through regulation of c-Cbl expression. Although diverse functions of c-Cbl have been widely studied, little is known about its gene regulation and further studies are needed to elucidate how ESDN regulates c-Cbl gene expression.

In conclusion, our data establish a novel regulatory role for ESDN in PDGF-induced signaling in VSMCs. The ESDN effect is, at least in part, mediated by c-Cbl which regulates PDGFR degradation and recycling. All of the experiments reported here used early passage primary human VSMCs. Similar ESDN functions have been observed in two non-primary cell lines: ESDN overexpression in 293T cells suppressed bromodeoxyuridine uptake (6), and its overexpression in gastric cancer cells inhibited colony formation in both anchorage-dependent and -independent cultures as well as cell invasion through the collagen matrix (31). ESDN expression has also been linked to acquisition of metastatic capability in lung cancer in vivo (6). None of these reports established the mechanisms of ESDN function. The observed effect of ESDN in regulating PDGFRβ signaling raises the possibility that ESDN modulates signaling of other
ESDN and Regulation of PDGF Signaling in VSMCs

receptor tyrosine kinases through similar mechanisms. Regulation of VSMC biology by ESDN raises the possibility that ESDN may serve as a therapeutic target for pathological states, such as post-angioplasty restenosis and graft arteriosclerosis where VSMC proliferation and migration play key roles in the pathogenesis. Demonstrating a similar inhibitory effect of ESDN on PDGFR signaling in other cell types and other signaling pathways would expand the potential clinical applications of ESDN to other proliferative disorders.

Acknowledgment—We thank Dr. George Tellides (Yale University) for experimental assistance and helpful suggestions.

REFERENCES

1. Owens, G. K., Kumar, M. S., and Wamhoff, B. R. (2004) Physiol. Rev. 84, 767–801.
2. Andrae, J., Gallini, R., and Betsholtz, C. (2008) Genes Dev. 22, 1276–1312.
3. Joazeiro, C. A., Wing, S. S., Huang, H., Leverson, J. D., Hunter, T., and Liu, Y. C. (1999) Science 286, 309–312.
4. Sadeghi, M. M., Esmailzadeh, L., Zhang, J., Guo, X., Asadi, A., Krassilnikov, A., Sasayama, S., Honjo, T., and Tashiro, K. (2001) J. Biol. Chem. 276, 29336–29347.
5. Koshikawa, K., Osada, H., Kozaki, K., Konishi, H., Masuda, A., Tatematsu, Y., Mitsudomi, T., Nakao, A., and Takahashi, T. (2002) Oncogene 21, 2822–2828.
6. Kawai-Kowase, K., and Owens, G. K. (2007) Am. J. Physiol. Cell Physiol. 292, 34105–34114.
7. Koshikawa, K., Osada, H., Kozaki, K., Konishi, H., Masuda, A., Tatematsu, Y., Mitsudomi, T., Nakao, A., and Takahashi, T. (2002) Oncogene 21, 2822–2828.
8. Mureebe, L., Nelson, P. R., Yamamura, S., Lawitts, J., and Kent, K. C. (1997) Surgery 122, 138–144.
9. Weber, D. S., Taniyama, Y., Rocic, P., Seshiah, P. N., Dechert, M. A., Gerthoffer, W. T., and Griendling, K. K. (2004) Circ. Res. 94, 1219–1226.
10. Erpel, T., and Courtneidge, S. A. (1995) Curr. Opin. Cell Biol. 7, 176–182.
11. Sambi, B. S., Hains, M. D., Waters, C. M., Connell, M. C., Willard, F. S., Kimple, A. J., Pyne, S., Siderovski, D. P., and Pyne, N. J. (2006) Cell Signal 18, 971–981.
12. Mori, S., Tanaka, K., Omura, S., and Saito, Y. (1995) J. Biol. Chem. 270, 29447–29452.
13. Mori, S., Heldin, C. H., and Claesson-Welsh, L. (1993) J. Biol. Chem. 268, 577–583.
14. Lewis, C. D., Olson, N. E., Raines, E. W., Reidy, M. A., and Jackson, C. L. (2001) Platelets 12, 352–358.
15. Jackson, C. L., Raines, E. W., Ross, R., and Reidy, M. A. (1993) Arterioscler. Thromb. Vasc. Biol. 13, 1218–1226.
16. Nakata, S., Fujita, N., Kitagawa, Y., Okamoto, R., Ogita, H., and Takai, Y. (2007) J. Biol. Chem. 282, 37815–37825.
17. Zhan, Y., Kim, S., Izumi, Y., Izumiya, Y., Nakao, T., Miyazaki, H., and Iwao, H. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 795–801.
18. Yoshida, T., Gan, Q., Shang, Y., and Owens, G. K. (2007) Am. J. Physiol. Cell Physiol. 292, C886–C895.
19. Mori, S., Heldin, C. H., and Claesson-Welsh, L. (1992) J. Biol. Chem. 267, 6429–6434.
20. Huang, M., Duhadaway, J. B., Prendergast, G. C., and Laury-Kleintop, L. D. (2007) Arterioscler. Thromb. Vasc. Biol. 27, 2597–2605.
21. Miyake, S., Mullan-Keilinon, K. P., Lill, N. L., Douillard, P., and Band, H. (1999) J. Biol. Chem. 274, 16619–16628.
22. Reddi, A. L., Ying, G., Duan, L., Chen, G., Dimri, M., Douillard, P., Druker, B. J., Naramura, M., Band, V., and Band, H. (2007) J. Biol. Chem. 282, 29336–29347.
23. Lechleider, R. J., Sugimoto, S., Bennett, A. M., Kashishian, A. S., Cooper, J. A., Shoelson, S. E., Walsh, C. T., and Neel, B. G. (1993) J. Biol. Chem. 268, 21478–21481.
24. Persson, C., Siévert, C., Bourdeau, A., Tremblay, M. L., Markova, B., Böhmer, D. F., Haj, F. G., Neel, B. G., Olson, A., Heldin, C. H., Rönnstrand, L., Ostman, A., and Hellberg, C. (2004) Mol. Cell. Biol. 24, 2190–2201.
25. Spencer, J. A., Hacker, S. L., Davis, E. C., Mecham, R. P., Knutsen, R. H., Li, D. Y., Gerard, R. D., Richardson, J. A., Olson, E. N., and Yanagisawa, H. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 2946–2951.
26. Schneller, M., Vuori, K., and Ruoslahti, E. (1997) EMBO J. 16, 5600–5607.
27. Sundberg, C., and Rubin, K. (1996) J. Cell Biol. 132, 741–752.
28. Welser, J. V., Lange, N., Singer, C. A., Elorza, M., Scowen, P., Keef, K. D., Gerthoffer, W. T., and Burkin, D. J. (2007) Circ. Res. 101, 672–681.
29. Juliano, R. L., Reddig, P., Alahari, S., Edin, M., Howe, A., and Aplin, A. (2004) Biochem. Soc. Trans. 32, 443–446.
30. Swaminathan, G., and Tsygankov, A. Y. (2006) J. Cell. Biol. 209, 21–43.
31. Kim, M., Lee, K. T., Jang, H. R., Kim, J. H., Noh, S. M., Song, K. S., Cho, J. S., Jeong, H. Y., Kim, S. Y., Yoo, H. S., and Kim, Y. S. (2008) Mol. Cancer Res. 6, 222–230.