

Wild-type LRP6 inhibits, whereas atherosclerosis-linked LRP6_{R611C} increases PDGF-dependent vascular smooth muscle cell proliferation

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Vascular smooth muscle cell (VSMC) proliferation is an important event in atherosclerosis and other vasculopathies. PDGF signaling is a key mediator of SMC proliferation, but the mechanisms that control its activity remain unclear. We previously identified a mutation in LDL receptor-related protein 6 (LRP6), LRP6_{R611C}, that causes early atherosclerosis. Examination of human atherosclerotic coronary arteries showed markedly increased expression of LRP6 and colocalization with PDGF receptor β (PDGFR- β). Further investigation showed that wild-type LRP6 inhibits but LRP6_{R611C} promotes VSMC proliferation in response to PDGF. We found that wild-type LRP6 forms a complex with PDGFR- β and enhances its lysosomal degradation, functions that are severely impaired in LRP6_{R611C}. Further, we observed that wild-type and mutant LRP6 regulate cell-cycle activity by triggering differential effects on PDGF-dependent pathways. These findings implicate LRP6 as a critical modulator of PDGF-dependent regulation of cell cycle in smooth muscle and indicate that loss of this function contributes to development of early atherosclerosis in humans.

human genetics | Wnt | cyclin D1 | ubiquitination | atherosclerotic diseases

Vascular smooth muscle cell (VSMC) proliferation is a major pathological component of atherosclerosis and many other vasculopathies. Activation of PDGF signaling is a key driver of VSMC proliferation, and both PDGF and PDGF receptor β (PDGFR- β) show increased expression in atherosclerotic lesions (1). Activation of PDGFR- β in response to endothelial injury induces VSMC proliferation and migration (2, 3). Conversely, inhibition of PDGF signaling reduces neointimal smooth muscle cell accumulation (4–8) and diminishes atherosclerotic burden (9, 10). Recent genetic evidence supports a causal role for increased VSMC proliferation in atherosclerosis (11).

Upon activation, PDGFR- β is phosphorylated, leading to phosphorylation of recombinant activated factor (RAF) (12), ERK1/2 (13), and JAK1/STAT1 (14), which then activate nuclear transcription factors leading to increased expression of cell-cycle regulators including cyclin D1 (15). The detailed mechanisms regulating expression and activation of PDGF and PDGFR- β are incompletely understood.

We recently identified a mutation in LDL receptor-related protein 6 (LRP6), LRP6_{R611C}, that underlies a Mendelian form of early atherosclerosis and metabolic syndrome. Mutation carriers have diffuse coronary artery and cerebrovascular disease and most often die of cardiovascular disease before age 50 y (16); they also show rapid disease progression after percutaneous coronary artery intervention. Although the genetic link between the identified LRP6 mutation and premature atherosclerosis is clear, the mechanisms by which the mutation imparts its effects have not yet been defined.

LRP6 is a member of the LDL receptor family and is known for its function as a membrane coreceptor for canonical Wnt signaling. This protein is expressed abundantly in smooth muscle cells and has been shown to play a role in cell-cycle activity in response to Wnt stimulation (17), raising the possibility

that LRP6 normally may play a role in regulation of VSMC proliferation. We report here an investigation of LRP6 and LRP6_{R611C} and their roles in VSMC proliferation.

Results

LRP6 Colocalizes with PDGFR- β and Is Expressed Abundantly in Atherosclerotic Lesions. We examined LRP6 expression in human coronary artery specimens from five persons with atherosclerosis and five healthy individuals without atherosclerotic coronary arteries. Immunohistochemical studies showed that in normal coronary arteries LRP6 was expressed primarily at very low levels in the intima. In contrast, LRP6 was much more expressed in the subintima and muscularis layer of all five atherosclerotic arteries than in normal coronary arteries (Fig. 1 and Fig. S1 A and B). Earlier studies of atherosclerotic lesions have shown that smooth muscle cells within these layers exhibit higher expression levels of PDGFR- β than do smooth muscle cells in normal coronary arteries (18, 19). LRP6 and PDGFR- β showed striking colocalization in both subintima and muscularis layers in all atherosclerotic coronary artery specimens. These findings motivated further studies to understand the effect of wild-type LRP6 (LRP6_{WT}) and LRP6_{R611C} on VSMC proliferation.

LRP6_{R611C} Attenuates Wnt-Dependent Expression of Cyclin D1 and VSMC Proliferation. A previous study suggested that LRP6 augments cyclin D1 expression and cell-cycle activity of VSMC in response to Wnt stimulation (17). We examined the impact of the LRP6_{R611C} mutation on these effects. We prepared adenoviral constructs expressing LRP6_{WT} or LRP6_{R611C} from the CMV promoter and showed that these constructs infected nearly 100% of primary human aortic smooth muscle cells (Methods). In response to Wnt3a stimulation, uninfected aortic smooth muscle cell exhibited a more than twofold increase in cyclin D1 expression and cell proliferation (Fig. 2 A and B). Infection with LRP6_{WT} further increased cell proliferation in response to Wnt stimulation. (The effect of LRP6_{WT} overexpression on cyclin D1 expression was not statistically significant.) In contrast, cells expressing LRP6_{R611C} showed no increase in cyclin D1 level and cell proliferation in response to Wnt. This finding is consistent with our previous observation that LRP6_{R611C} impairs Wnt signaling (16) and indicates that the atherosclerosis seen in LRP6_{R611C} mutation carriers cannot be explained by increased Wnt-dependent smooth muscle proliferation.

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The authors declare no conflict of interest.

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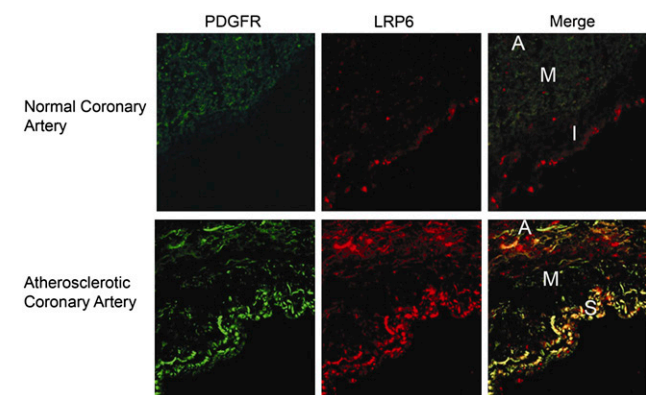


Fig. 1. PDGFR- β and LRP6 expression in normal and atherosclerotic coronary arteries. Immunofluorescent staining in a cross-section of a normal coronary artery shows that LRP6 and PDGFR- β are expressed at very low levels in the intima (I) and muscularis (M) layers, respectively (Upper). In the cross-section of the atherosclerotic coronary artery, LRP6 and PDGFR- β are highly expressed and colocalized in the subintima (S) and the muscularis (M) layers (Lower) ($n = 5$). A, adventitia.

LRP6_{WT} Inhibits but LRP6_{R611C} Augments Proliferation in Response to PDGF- β . PDGF is a key regulator of VSMC proliferation. Because LRP6 and PDGFR- β colocalize and show increased expression in atherosclerotic lesions versus coronary arteries, we examined the effect of LRP6_{WT} and LRP6_{R611C} on cellular proliferation in response to PDGF- β . Consistent with earlier findings (19), control VSMC (infected with empty adenovirus vector) showed a 45% increase in cellular proliferation after stimulation with PDGF- β . This proliferative response to PDGF- β stimulation was significantly blunted in smooth muscle cells overexpressing LRP6_{WT} (Fig. 3A). In marked contrast, in the presence of LRP6_{R611C}, PDGF increased cellular proliferation by 80%, significantly more than in either LRP6_{WT} or control. This LRP6_{R611C}-induced increase in proliferation is associated with a proportional increase in the expression level of cyclin D1 (Fig. 3B).

VSMC express endogenous LRP6. If endogenous LRP6 inhibits the response to PDGF, knock down of LRP6 with shRNA-containing lentivirus should result in increased proliferation. This treatment resulted in a significantly higher proliferation rate than seen in cells that express scrambled control shRNA (Fig. 3C). These findings collectively demonstrate that LRP6 negatively regulates cellular proliferation in response to PDGF signaling and that LRP6_{R611C} has dominant-negative effects on this inhibition.

LRP6_{WT} and LRP6_{R611C} Have Opposite Effects on PDGFR- β Expression and Activation. To investigate the mechanism of the different effects of LRP6_{WT} and LRP6_{R611C}, we examined PDGFR- β expression and activation in human aortic smooth muscle cells infected with empty adenovirus vectors and vectors expressing LRP6_{WT} and LRP6_{R611C}. Cells expressing LRP6_{WT} exhibited a dramatic decrease in levels of PDGFR- β protein; in contrast, LRP6_{R611C} induced little if any decrease in PDGFR- β (Fig. 4A). This wild-type effect was dose dependent (Fig. 4B) and was absent in the presence of LRP6_{R611C} (Fig. S24). In contrast, knocking down LRP6 levels by RNA interference raised PDGFR- β levels (Fig. 4C). The dramatic decrease in PDGFR- β protein level with increased LRP6_{WT} was not accompanied by a change in PDGFR- β mRNA level (Fig. S2B). These results demonstrate that LRP6_{WT} expression causes reduction of PDGFR- β and that LRP6_{R611C} impairs this effect.

LRP6 Forms a Complex with PDGFR- β and Promotes Its Degradation. To determine whether LRP6 and PDGFR- β form a complex, we performed coimmunoprecipitation in cells infected with a modest

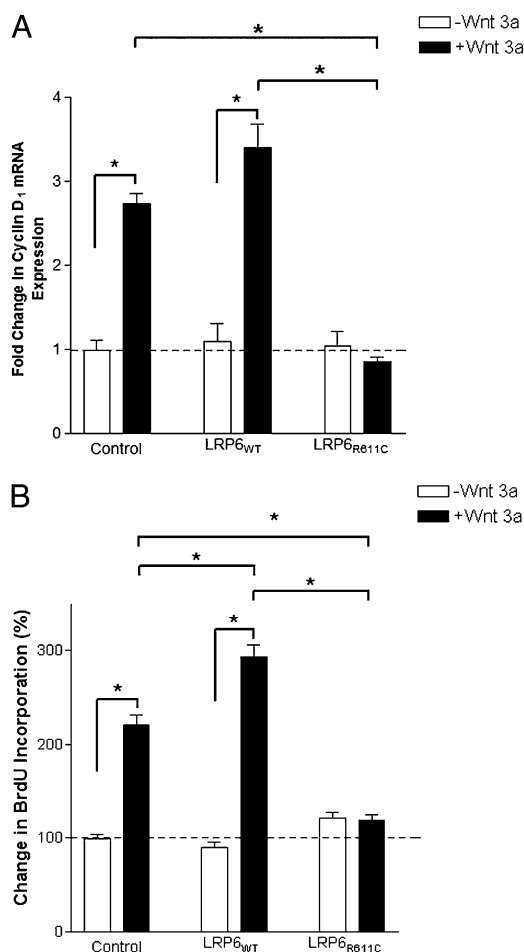


Fig. 2. Effect of LRP6_{WT} and LRP6_{R611C} on cyclin D1 expression and cell proliferation. (A) After 12-h stimulation with Wnt3a, mRNA expression levels of cyclin D1 were about threefold lower in cells expressing LRP6_{R611C} than in uninfected cells ($P < 0.0001$) or cells expressing LRP6_{WT} ($P < 0.001$). (B) Wnt3a stimulated the proliferation rate in aortic smooth muscle cells overexpressing LRP6_{WT} and LRP6_{R611C}. Percent BrdU incorporation in cells expressing LRP6_{R611C} after stimulation with Wnt3a remained unchanged and was considerably lower than in cells infected with empty vector (Control) ($P < 0.001$) or cells expressing LRP6_{WT} ($P < 0.001$).

multiplicity of infection of virus expressing LRP6 tagged with HA (1,000 virus particles per cell). Proteins from cell lysates were immunoprecipitated with anti-HA or anti-PDGFR- β antibodies followed by Western blotting with anti-PDGFR- β or anti-HA antibodies, respectively. Immunoprecipitation in both directions showed that LRP6_{WT} and LRP6_{R611C} are in a complex with PDGFR- β (Fig. 5A). The specificity of this interaction was demonstrated by the lack of coimmunoprecipitation of PDGFR- β with IgG or Niemann-Pick disease, type C2 (NPC2).

PDGFR- β levels are regulated by ubiquitination (20) and proteosomal or lysosomal degradation (21). Accessory proteins, such as LRP1, have been shown to target PDGFR- β for degradation (22, 23), suggesting that LRP6 might play a similar role. Cells expressing LRP6_{WT} exhibit significantly higher PDGFR- β ubiquitination following incubation with the proteasome inhibitor MG132 than cells expressing LRP6_{R611C} or infected with empty vector (Fig. 5B).

Reduced levels of PDGFR- β in the presence of LRP6_{WT} were rescued only partially by the proteosomal inhibitor MG132 (Fig. S34). In contrast, when cells were treated with the lysosomal inhibitor NH₄Cl, we observed nearly complete rescue of PDGFR- β

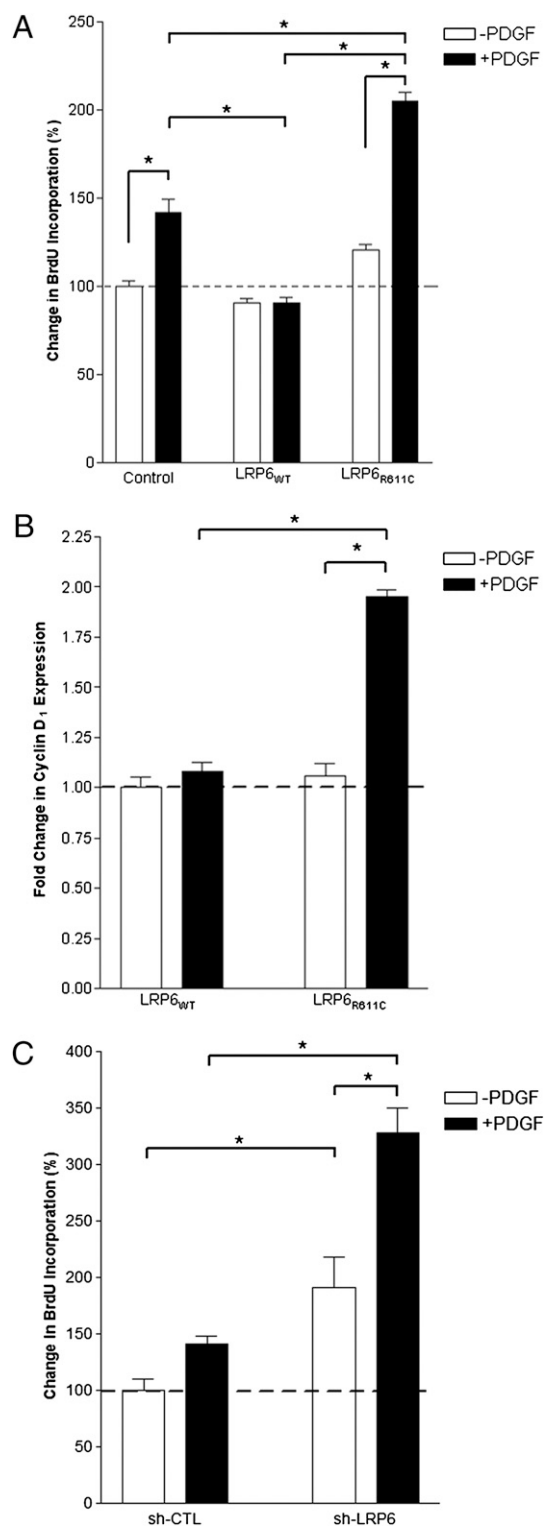


Fig. 3. Increased cell-cycle activity in human aortic smooth muscle cells expressing LRP6^{R611C} or LRP6 shRNA. (A) Percent change in BrdU incorporation is considerably higher in cells expressing LRP6^{R611C} in response to PDGF and is considerably lower in cells expressing LRP6^{WT} cells ($P < 0.05$) than in cells infected with empty vector ($P < 0.001$). (B) Fold change in cyclin D₁ expression levels. After stimulation with PDGF, cyclin D₁ expression levels were significantly increased in cells expressing LRP6^{R611C} ($P < 0.01$) but remained unchanged in cells expressing LRP6^{WT}. (C) Increased proliferation rate in cells infected with LRP6-specific shRNA (sh-LRP6). RNA interference significantly increased the cell proliferation rate both without ($P < 0.05$) and with PDGF stimulation compared with scrambled shRNA (sh-CTL) ($P < 0.001$).

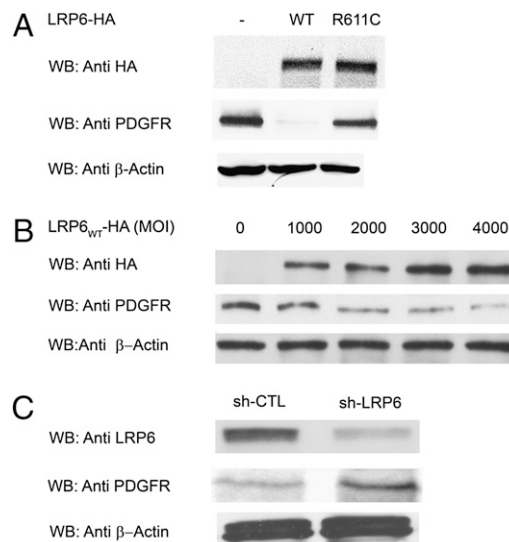


Fig. 4. Expression levels of PDGFR- β in human aortic smooth muscle cells expressing LRP6^{WT} and LRP6^{R611C}. (A) LRP6^{WT} significantly reduces PDGFR- β expression levels ($P = 0.001$). This effect is diminished significantly in the presence of LRP6^{R611C}. (B) Dose effect of LRP6^{WT} on endogenous PDGFR- β expression. There is an inverse relationship between the multiplicity of infection of viral particles expressing LRP6^{WT}-HA2 and PDGFR- β expression. (C) LRP6 knock down by RNA interference (shLRP6). Knocking down LRP6 by RNA interference raised PDGFR- β levels ($P = 0.01$).

levels (Fig. 5C). This result suggests that lysosomal degradation plays a greater role in degradation of PDGFR- β in these cells. Immunostaining revealed that expression of LRP6^{WT} but not LRP6^{R611C} led to redistribution of a substantial fraction of PDGFR- β to an intracellular compartment that costains with early endosomal antigen 1 (EEA1) and the lysosomal marker lysosomal-associated membrane protein 2 (LAMP2), consistent with a substantial fraction of this intracellular PDGFR- β being in the lysosomal pathway (Fig. S3B–D). Together these findings indicate that LRP6 is in a complex with PDGFR- β and promotes its degradation.

LRP6^{WT} and LRP6^{R611C} Have Differential Effects on PDGF Signaling Peptides. PDGF exerts its proliferative effects on VSMC largely through activation of ERK1/2 (24) and STAT1 (25). Investigation of the PDGF signaling components revealed that the anti-proliferative effect of LRP6 is accounted for by reduced activation of ERK1/2 (p42, and p44) and the JAK–STAT pathway (Fig. S4A–C). LRP6^{WT} reduces phosphorylation of ERK1/2, JAK1, and STAT1 after stimulation with PDGF- β . In contrast, LRP6^{R611C} significantly increases JAK1 and STAT1 serine and tyrosine phosphorylation (Fig. 5D).

Discussion

LRP6 is well known as a coreceptor with frizzled proteins in the Wnt signaling pathway; however, its Wnt-independent roles have been largely unexplored. LRP6 is abundantly expressed in human VSMC and, as we show here, attenuates PDGF signaling by promoting ubiquitination and degradation of PDGFR- β , thereby reducing expression of cyclin D₁ and VSMC proliferation. Knock down of endogenous LRP6 by RNA interference shows the opposite effect, indicating that the effect of LRP6 on PDGFR ubiquitination is specific. PDGF is a key regulator of VSMC proliferation (1), which plays a critical role in the pathogenesis of atherosclerosis in both human and animal models (11). The increased expression of both PDGFR- β and LRP6 and their colocalization in atherosclerotic lesions is consequently of considerable interest, suggesting that LRP6 plays an inhibitory and

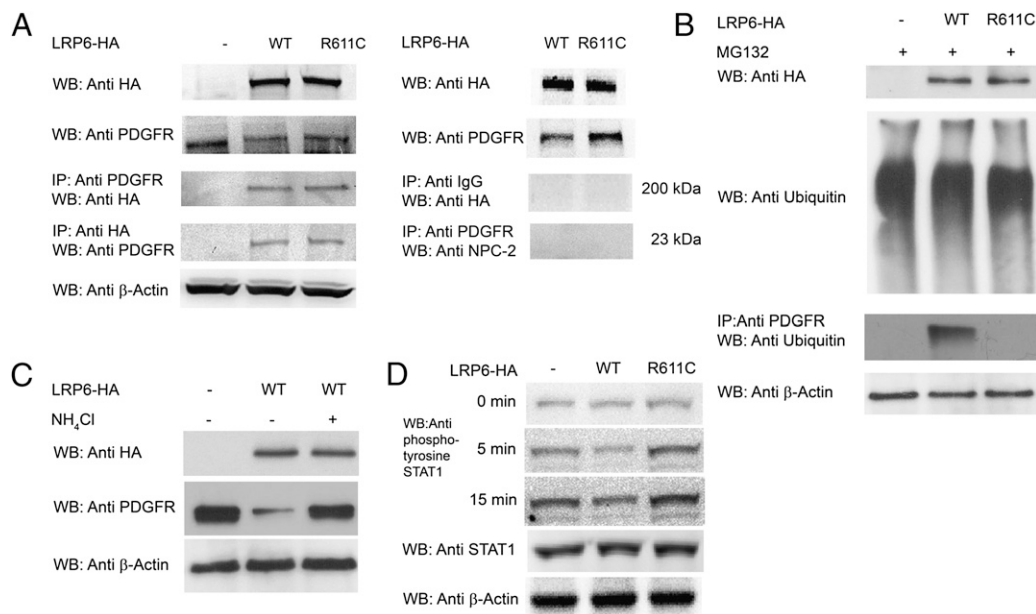


Fig. 5. LRP6_{WT} and LRP6_{R611C} form complexes with PDGFR- β . (A) Proteins from cell lysates were immunoprecipitated with either anti-HA or anti-PDGFR- β antibodies followed by Western blotting with either anti-PDGFR- β or anti-HA antibodies, respectively. LRP6_{WT} and LRP6_{R611C} but not IgG or NPC2 (used as control) coimmunoprecipitated with PDGFR- β . (B) LRP6_{WT} ubiquitinates PDGFR- β . Ubiquitination of PDGFR- β following MG132 treatment in cells overexpressing LRP6_{R611C} or LRP6_{WT} and in cells infected with empty vector was compared. LRP6_{WT} but not LRP6_{R611C} ubiquitinates PDGFR- β . (C) Lysosomal degradation of PDGFR- β . The lysosomal inhibitor NH₄Cl rescues reduced expression of PDGFR- β ($P = 0.001$). (D) LRP6_{WT} reduces and LRP6_{R611C} increases STAT1 tyrosine phosphorylation. After PDGF stimulation, tyrosine phosphorylation of STAT1 is significantly higher in cells expressing LRP6_{R611C} ($P < 0.001$) and is significantly lower in cells expressing LRP6_{WT} ($P < 0.001$) than in cells transfected with empty plasmid.

potentially beneficial role in these lesions. Impairment of this function probably contributes to accelerated atherosclerosis in carriers of the LRP6_{R611C} mutation.

These effects of LRP6 are similar to those of LRP1 (26). Although LRP1's effect on PDGF signaling is believed to be regulated by ligand interactions, the mechanisms by which LRP6's effects are regulated, as well as the factors inducing increased LRP6 expression in atherosclerotic lesions, are unknown and will be of interest in further investigations.

Of particular interest is our demonstration that the LRP6_{R611C} mutation linked to a Mendelian form of premature coronary artery disease has dominant-negative effects on the inhibition of PDGFR- β . Thus, there is greater proliferation in the presence of this mutation than in normal cells. In conjunction with the increased LRP6 expression in atherosclerotic lesions, this observation can account for the observed dominant transmission of the trait and a contribution of this mutation to atherosclerosis via increased VSMC proliferation.

These findings do not directly explain the metabolic consequences in patients harboring this mutation; however, the observed promiscuity of LRP6 interactions raises the possibility of effects not only on the Wnt signaling pathway but with other signaling pathways involved in metabolic functions. We anticipate that further studies of the effects of wild-type and mutant LRP6 on metabolism will be illuminating.

Materials and Methods

Immunohistochemical Studies of Normal and Sporadic Atherosclerotic Coronary Arteries. Sectioning and staining of the specimens with antibodies against LRP6 (Abgent) and PDGFR- β (Santa Cruz) were carried out by the Surgical Pathology Service of the Yale University Department of Pathology using 1:100 diluted LRP6 antibody, 1:50 diluted PDGFR- β antibody, and 1:500 diluted Alexa Fluor 488 and Alexa Fluor 568 fluorescence-conjugated secondary antibodies.

Viral Infection of Aortic Smooth Muscle Cells with Adenovirus. Human aortic smooth muscle cells were cultured in M199 medium containing 20% bovine serum to 90% confluence and were infected with replication-defective cytomegalovirus promoter LRP6-HA2, LRP6_{R611C}-HA2, or empty adenovirus vectors created by Viraguest. Efficiency of gene transfer was assessed by concomitant infection with Adv-GFP and by epifluorescence microscopy.

Cyclin D1 Expression Levels. Cells were infected with HA-LRP6_{WT}, HA-LRP6_{R611C}, or empty adenovirus as described. Seventy-two hours after infection cells were subjected to starvation (DMEM + 0.2% FBS) for 24 h. Wnt3a (25 ng/mL) or PDGF-BB (30 ng/mL) was added for 12 h. Then RNA was extracted using Trizol (Invitrogen), and cDNA was produced using SuperScript III reverse transcription kits (Invitrogen). Expression levels were reexamined by real-time PCR experiments as described (27).

Analysis of the Expression and Activation of PDGFR- β and Its Downstream Peptides in Human Aortic Smooth Muscle Cells. PDGF-BB (Millipore) was added to the medium at a final concentration of 30 ng/mL at 5, 15, and 30 min following stimulation, cell extracts were prepared with RIPA buffer containing protease inhibitor (Roche) and Halt Phosphatase Inhibitor Mixtures (Thermo Scientific) and were subjected to immunoblotting with antibodies against PDGFR- β (Cell Signaling), HA (Cell Signaling), and total and phospho-ERK1/2, JAK1, p-JAK1, STAT1, phosphoserine STAT1, and phosphotyrosine STAT1 (Cell Signaling).

Cells were infected with incremental doses of viral particles containing LRP6 (0, 1,000, 2,000, 3,000, and 4,000 virus particles per cell) to investigate the dose effect of LRP6 on PDGFR- β expression. Lysates were prepared and subjected to immunoblotting with PDGFR- β antibody as described.

LRP6 shRNA (SMARTvector 2.0 Lentiviral shRNA technology) were purchased from Dharmacon and were reverse-infected according to the manufacturer's instructions.

Investigation of PDGFR- β Degradation by LRP6. Cells were infected with HA-LRP6_{WT}, HA-LRP6_{R611C}, or empty adenovirus as described. Cells were lysed in a nondenaturing cell lysis buffer (Cell Signaling) and were subjected to immunoprecipitation with PDGFR or HA antibodies. Precipitated proteins were subjected to immunoblot analysis with anti-HA, anti-NPC2 (Santa Cruz Biotechnology), and immobilized mouse IgG (Cell Signaling) antibodies.

For PDGFR- β mRNA expression levels, RNA was extracted from infected cells, and real-time PCR experiments were carried out as described.

For proteosomal degradation studies, proteosomal inhibitor MG132 (Sigma) was added to the cell medium 72 h after infection to a final concentration of 2 μ M for 2 h. Cell extracts were subjected to immunoprecipitation with PDGFR antibody, and precipitated proteins were subjected to immunoblot analysis with anti-ubiquitin (Cell Signaling). For lysosomal degradation studies, NH₄Cl was added to the cell medium to a final concentration of 20 mM for 12 h.

For immunofluorescent studies, cells were placed in 24-well plates containing coverslips and were infected as described. Seventy-two hours later, cells were fixed by 4% paraformaldehyde and were permeabilized in 0.1% Triton X-100 and blocked with 3% BSA in PBS after appropriate washes and incubated with 1:100 diluted PDGFR- β , HA, EEA1 (Abcam), and LAMP2 (Abcam) overnight at 4 °C. Cells subsequently were incubated with 1:500 diluted Alexa Fluor 488 and Alexa Fluor 568 fluorescence-conjugated secondary antibodies (Invitrogen) at room temperature and were mounted with Vectashield (Vector Laboratories). Specimens were examined by a Zeiss LSM510 confocal microscope using excitation and emission filters at 488 nm and 522 nm, respectively.

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