Deletion of LR11 Attenuates Hypoxia-Induced Pulmonary Arterial Smooth Muscle Cell Proliferation With Medial Thickening in Mice

Le Jiang,* Hakuoh Konishi,* Fariz Nurwidya, Kimio Satoh, Fumiyuki Takahashi, Hiroyuki Ebinuma, Kengo Fujimura, Kiyoshi Takasu, Meizi Jiang, Hiroaki Shimokawa, Hideaki Bujo, Hiroyuki Daida

Objective—We aimed to determine whether LR11 (low-density lipoprotein receptor with 11 binding repeats) is a potential key regulator of smooth muscle cell (SMC) proliferation during the progression of hypoxia-induced medial thickening in mice and whether sLR11 (soluble LR11) can serve as a biomarker in patients with pulmonary arterial hypertension.

Approach and Results—The role of LR11 in pulmonary arterial hypertension was investigated using mouse and cell models of induced hypoxia. The expression of LR11 and of hypoxia-inducible factor-1α was significantly increased in lung tissues from C57Bl/6 mice after 3 weeks of exposure to hypoxia compared with normoxia. Serum sLR11 levels were also increased. Physiological and histochemical analyses showed that increased right ventricular systolic pressure, right ventricular hypertrophy, and medial thickening induced under hypoxia in wild-type mice were attenuated in LR11$^{-/-}$ mice. The proliferation rates stimulated by hypoxia or platelet-derived growth factor-BB were attenuated in SMC derived from LR11$^{-/-}$ mice, compared with those from wild-type mice. Exogenous sLR11 protein increased the proliferation rates of SMC from wild-type mice. The expression of LR11 and hypoxia-inducible factor-1α was increased in cultured SMC under hypoxic conditions, and hypoxia-inducible factor-1α knockdown almost abolished the induction of LR11. Serum sLR11 levels were significantly higher in patients with, rather than without, pulmonary arterial hypertension. sLR11 levels positively correlated with pulmonary vascular resistance and mean pulmonary arterial pressure.

Conclusions—LR11 regulated SMC proliferation during the progression of hypoxia-induced medial thickening in mice. The findings obtained from mice, together with those in humans, indicate that sLR11 could serve as a novel biomarker that reflects the pathophysiology of proliferating medial SMC in pulmonary arterial hypertension. **(*Arterioscler Thromb Vasc Biol*. 2016;36:1972-1979. DOI: 10.1161/ATVBAHA.116.307900.)**

Key Words: atherosclerosis ■ biomarkers ■ disease progression ■ LR11/SorLA ■ pulmonary hypertension ■ vascular smooth muscle

Pulmonary arterial hypertension (PAH) is a critical condition with a median survival of 2.8 years if left untreated.[1] Novel drugs with vasodilator action, such as endothelin-1 receptor antagonist, phosphodiesterase-5 inhibitors, soluble guanylate cyclase stimulator, and prostacyclin analogues, have recently improved symptoms, exercise capacity, and hemodynamics among patients with PAH.[2–5] Although the initial phases of PAH are clinically silent, the EARLY study showed that mildly symptomatic PAH when left untreated progressively deteriorates both clinically and hemodynamically, regardless of exercise capacity.[2] Therefore, the early detection of initially silent PAH pathophysiology is important to improve treatment outcomes. In this context, specific biomarkers of the initial progression of the disease are needed, and BNP/N-terminal pro b-type natriuretic peptide has served as a sensitive marker in this capacity.[6] Thus, novel circulating molecules based on a pathophysiology that differs from that of BNP/N-terminal pro b-type natriuretic peptide are required to increase the ability to detect initial disease progression and improve individual treatment strategies. One candidate marker might be a circulating molecule that represents the proliferation of smooth muscle cells (SMC) during the progress of medial thickening. These cells proliferate abnormally under crosstalk with dysfunctional endothelial cells and other components of the

Received on: June 8, 2015; final version accepted on: July 19, 2016.

From the Department of Cardiovascular Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan (L.J., H.K., K.T., H.D.); Department of Respiratory Medicine, Juntendo University Graduate School of Medicine, Tokyo, Japan (F.N., F.T.); Department of Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan (K.S., H.S.); Tsukuba Research Institute, Sekisui Medical Co Ltd, Ryugasaki, Japan (H.E., K.F.); and Department of Clinical-Laboratory and Experimental-Research Medicine, Toho University Sakura Medical Center, Sakura, Japan (M.J., H.B.).

This manuscript was sent to William Sessa, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

*These authors contributed equally to this article.

The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.116.307900/-/DC1.

Correspondence to Hakuoh Konishi, MD, PhD, Department of Cardiovascular Medicine, Juntendo University Graduate School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan. E-mail konishi@juntendo.ac.jp

Nonstandard Abbreviations and Acronyms	
BNP	brain natriuretic peptide
HIF-1α	hypoxia-inducible factor-1α
LR11	low-density lipoprotein receptor with 11 binding repeats
PAH	pulmonary arterial hypertension
PASMC	pulmonary arterial smooth muscle cells
SMC	smooth muscle cells
VSMC	vascular smooth muscle cells
WT	wild type

pulmonary vascular wall including myofibroblasts, pericytes, and circulating immune cells, and thus, a complicated mechanism underlies the SMC proliferation, one of the key features in PAH pathogenesis.[7] Vascular smooth muscle cells (VSMCs) proliferate in the media of the pulmonary artery of patients with PAH,[8] and several growth factors, such as platelet-derived growth factor, act as potent mitogens and chemoattractants for VSMC and also cause vascular remodeling.[9]

LR11 (also called SorLA or SORL1) is a low-density lipoprotein receptor[10] that is expressed in intimal SMCs during the development of atherosclerosis.[11–13] LR11 released in a soluble form (sLR11) from the intimal SMC membrane by proteolytic shedding during the phase of rapid SMC proliferation induces SMC migration.[11,14] Recent clinical studies have suggested that serum sLR11 could serve as a circulating marker of intimal SMC and reflect cell functions, particularly those of medial SMC proliferation and migration after phenotype alteration.[15]

The present study investigated the functional significance of sLR11 as a regulator of SMC in medial thickening, which is the typical pathophysiology of PAH, using animal models. We then studied the potential of LR11 as a novel biomarker for pathologically proliferating medial SMCs in patients with PAH.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Hypoxia-Induced LR11 Expression in Murine Lungs

We assessed LR11 involvement in hypoxia-induced damage to the pulmonary arteries of mice. Western blotting showed significantly increased HIF-1α expression (0.33±0.05 versus 0.53±0.04 $P<0.05$; Figure 1A and 1D), LR11 expression (0.25±0.03 versus 0.65±0.04; $P<0.05$; Figure 1A and 1B), and serum sLR11 levels (Figure 1C) in mice under hypoxia, compared with normoxia. These results indicated that hypoxia induced LR11 expression and the release of sLR11 from the lungs, together with hypoxia-induced intracellular signals via HIF-1α. Therefore, LR11 might be involved in the development of PAH in response to hypoxia.

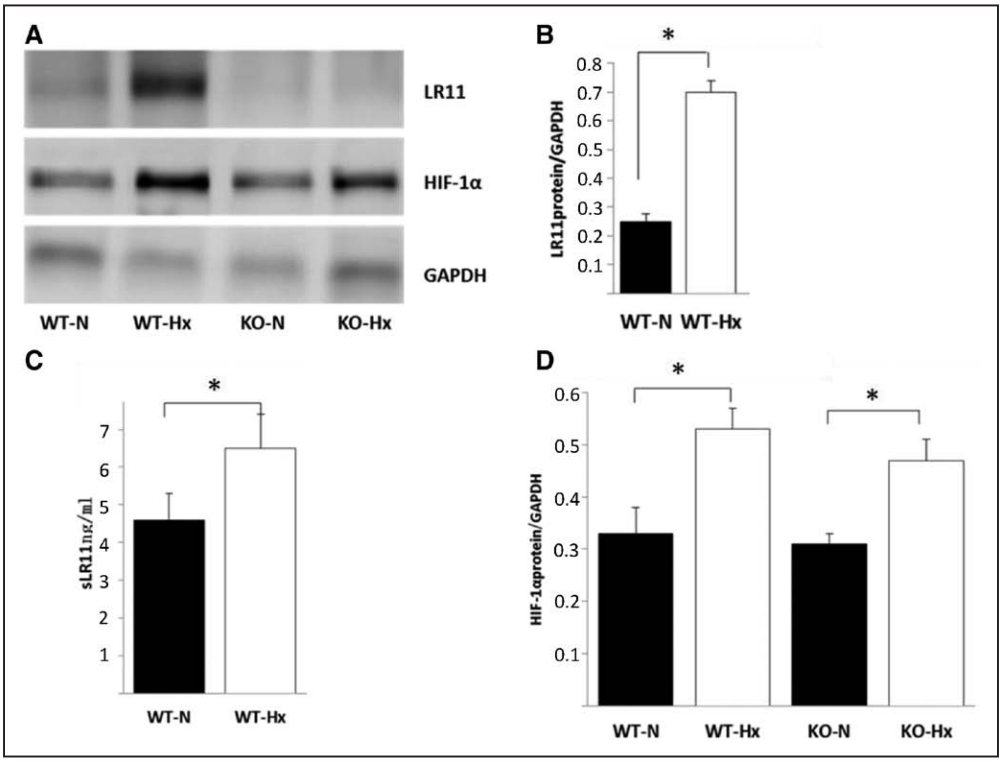

Figure 1. Western blots of LR11 (low-density lipoprotein receptor with 11 binding repeats) and hypoxia-inducible factor-1α (HIF-1α) protein expression in lungs of mice exposed to normoxia or hypoxia. **A** and **B**, LR11 protein expression significantly increased in lungs of wild-type (WT) mice exposed to hypoxia (Hx) compared with normoxia (N). **A** and **D**, Expression of HIF-1α protein significantly increased in WT mice under hypoxia compared with normoxia (0.25±0.03 vs 0.65±0.04; *$P<0.05$). Lung HIF-1α levels did not significantly differ between WT and LR11$^{-/-}$ mice under hypoxia. **C**, Levels of sLR11 (soluble LR11) increased in WT mice after 3 wk of hypoxia compared with those under normoxia (4.6±0.7 vs 6.5±1.0 ng/mL; *$P<0.05$). Data in bar graphs are expressed as means±SD (n=5). Statistical significance was determined with unpaired Student *t* test. KO indicates knockout.

LR11$^{-/-}$ Mice Are Resistant to Induction of Pulmonary Hypertension

We investigated the role of LR11 in the development of PAH in LR11$^{-/-}$ mice that were placed under hypoxia for 21 days to induce experimental PAH.[16] Lung HIF-1α levels did not significantly differ between wild-type (WT) and LR11$^{-/-}$ mice under hypoxia (Figure 1A and 1D). The right ventricular systolic pressure and ratio of RV/LV+IVS (right ventricle/left ventricle+interventricular septum) were significantly lower in LR11$^{-/-}$ than in WT mice (28.3±2.2 versus 33.3±2.2 mm Hg; P<0.05 and 30.9±2.2% versus 36.4±2.4%; P<0.05, respectively; Figure 2A through 2C). These results indicated that LR11-deficient mice are highly resistant to developing PAH induced by hypoxia.

Index of Medial Wall Thickness Is Reduced in LR11$^{-/-}$ Mice

Histological findings showed that the index of medial wall thickness in small pulmonary arteries was significantly lower in LR11$^{-/-}$ than in WT mice (3.6% ± 0.5% versus 5.7% ± 0.4%; P<0.05; Figure 3A and 3B). The ratios of arteries categorized as muscular, partly muscular, and nonmuscular did not differ between LR11$^{-/-}$ and wild-type mice under normoxia. However, the ratios of partly muscular and muscular arteries in LR11$^{-/-}$ mice were significantly decreased, whereas the ratios of nonmuscular arteries in LR11$^{-/-}$ mice were significantly increased compared with those in WT mice under hypoxia (n=4; *P<0.05; Figure 3C). The results of an immunohistochemical comparison using anti-CD45 antibody, which is a common antigen for leukocytes, revealed significantly fewer CD45-positive cells in LR11$^{-/-}$, than in WT mice. These findings suggested that decreased inflammatory cell infiltration, in addition to decreased pulmonary arterial smooth muscle cell (PASMC) proliferation, is involved in the decreased medial thickening in the absence of LR11. All these findings indicated that LR11-deficient mice are highly resistant to the development of medial thickening induced by hypoxia (Figure 3C).

LR11 Is a Potential Key Regulator of SMC Proliferation Under Hypoxia

Because sLR11 has been shown to be a phenotype regulator of contractile SMC to synthetic SMC, and highly associated with intimal thickening after the injury of femoral arteries in mice,[17] we assessed the proliferation of PASMC to determine the pathological role of sLR11 in the development of PAH in model mice. Proliferation rates under hypoxia (1% O_2 and 5% CO_2) for 48 hours and in the presence of platelet-derived growth factor-BB (20 ng/mL) were significantly decreased in PASMC isolated from LR11$^{-/-}$, compared with those from wild-type mice (0.18±0.02 versus 0.45±0.05 nm; P<0.05 and 0.16±0.01 versus 0.45±0.07 nm; P<0.05, respectively; Figure 4). In contrast, the proliferation rates of PASMC from wild-type mice were significantly increased in the presence, compared with the absence, of 10 ng/mL of sLR11 (0.19±0.01 versus 0 0.42±0.02 nm; P<0.05). These results suggested that decreased PASMC proliferation contributes at least in part to the mechanism underlying the increased resistance of LR11$^{-/-}$ mice to hypoxia-induced PAH.

Hypoxia-Induced LR11 Expression in Cultured Human Pulmonary Arterial Smooth Muscle Cells Is Dependent on HIF-1α Pathway

The above findings of LR11$^{-/-}$ and WT mice suggested that LR11, a regulator of SMC proliferation and migration that

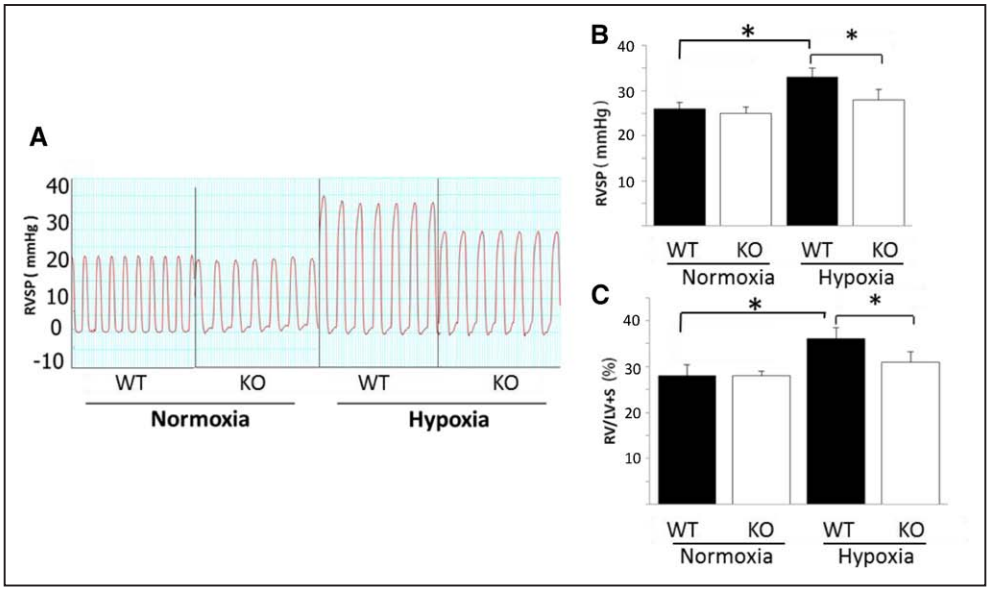

Figure 2. Effects of LR11 (low-density lipoprotein receptor with 11 binding repeats) deletion on hypoxia-induced pulmonary hypertension and of chronic hypoxia on right ventricular systolic pressure and right ventricular hypertrophy. **A**, Representative traces of right ventricular systolic pressure (RVSP) of wild-type (WT) and LR11$^{-/-}$ mice exposed to normoxia and hypoxia for 3 wk. **B**, Pulmonary hypertension did not develop in LR11$^{-/-}$ mice compared with WT mice (28.3±2.2 vs 33.3±2.2 mm Hg;*P<0.05). **C**, Ratios of right to left ventricular weight plus septal weight RV/(LV+S) of WT and LR11$^{-/-}$ mice exposed to normoxia and hypoxia for 3 wk. Values for RV/LV+S in LR11$^{-/-}$ mice are significantly decreased compared with those in WT mice (30.9±2.2% vs 36.4±2.4%; *P<0.05; n=8 mice per group). Data in bar graphs are expressed as means±SD (n=8). Statistical significance was determined with Tukey honestly significant difference.

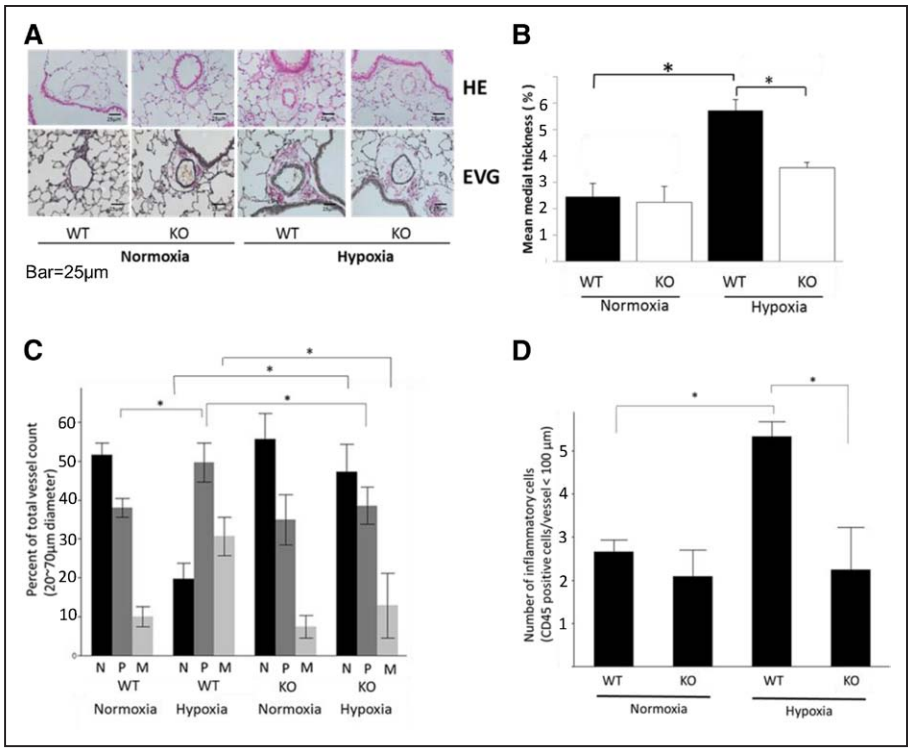

Figure 3. LR11 (low-density lipoprotein receptor with 11 binding repeats) deletion ameliorates vascular remodeling under hypoxic conditions. **A**, Ratio (%) of wall thickness of small pulmonary arteries exposed to chronic hypoxia. Representative photomicrographs of vascular remodeling in distal arterioles of lungs exposed to hypoxia for 3 wk. Lung sections were stained with hematoxylin eosin (HE) and Elastic van Gieson. Magnification, ×40; bar=25 μm. **B**, Medial wall thickness index (%WT) in distal arterioles (diameter, 50–100 μm) of lungs from LR11$^{-/-}$ and wild-type (WT) mice significantly differed after exposure to hypoxia for 3 wk (3.6±0.5% vs 5.7±0.4%; P<0.05). Bar graphs show data expressed as means±SD (n=8). *P<0.05. **C**, Ratios of 3 arteries categorized as muscular, partly muscular, and nonmuscular, did not differ between LR11$^{-/-}$ and WT mice under normoxia. Ratios of partly muscular and muscular arteries in LR11$^{-/-}$ mice were significantly decreased and those of nonmuscular arteries were significantly increased in LR11$^{-/-}$ compared with WT mice under hypoxia (n=4 each, *P<0.05). **D**, Number of CD45^{+} cells in distal pulmonary artery adventitia was significantly reduced in LR11$^{-/-}$ compared with WT mice after exposure to hypoxia. Number of CD45^{+} cells did not differ between LR11$^{-/-}$ and WT mice under normoxia (n=4 each;*P<0.05. Data in bar graphs are expressed as means±SD.). Statistical significance was determined with Tukey honestly significant difference. EVG indicates Elastica van Gieson.

causes vascular intimal thickening after injury,[17] is involved in the progression of PAH as an effector gene under HIF1α-mediated intracellular signals. We therefore assessed LR11 and HIF-1α expression in PASMC after incubation for 24, 48, 72, and 96 hours under hypoxic conditions (1% O_2 and 5% CO_2). The expression of LR11 peaked at 48 hours under hypoxia compared with normoxia (0.84±0.02 versus 0.31±0.03; P<0.05; Figure 5A and 5B). The transient increase in LR11 at this time point was similar to that of HIF-1α (0.92±0.05 versus 0.41±0.04; P<0.05; Figure 5A and 5B). Thus, we analyzed the effect of HIF1α knockdown on LR11 expression after hypoxia. The sharp increase in LR11 expression at 48 hours was almost completely abrogated in HIF1α-knockdown PASMC under hypoxia (Figure 5C and 5D). These results indicated that LR11 expression is induced via HIF-1α signaling after hypoxia and that it might cause increased PASMC migration and proliferation.

Serum sLR11 Levels Are Increased in Patients With PAH

We investigated whether sLR11 could serve as a marker of PAH in patients because the findings in vitro and in vivo indicated that LR11 is important for PAH progression in mice. We prospectively enrolled 20 consecutive patients with suspected pulmonary hypertension (Figure 6) and then compared serum sLR11 levels in patients with and without confirmed PAH whose age, sex, and World Health Organization functional class did not significantly differ (Table 1). Most patients had World Health Organization functional class II PAH. Connective tissue diseases were associated with PAH in 9 of 11 patients and 2 had portopulmonary hypertension. All of the patients without PAH had connective tissue diseases. Levels of sLR11 were significantly higher in patients with, rather than without, PAH (14.2±4.5 versus 9.2±4.1 ng/mL; P=0.019), whereas levels of BNP and uric acid did not significantly differ (Table 1). Pearson correlation coefficient analyses showed that the sLR11 levels positively correlated with mean pulmonary arterial pressure (r=0.633; P=0.003) and pulmonary vascular resistance (r=0.580; P=0.007) among all variables (Table 2). Thus, increased sLR11 levels in patients may reflect the pathological status of SMC in PAH.

Discussion

The major finding of this study is that LR11 regulated medial thickening during the process of vascular remodeling in mice with PH. A deletion of LR11 did not result in pulmonary

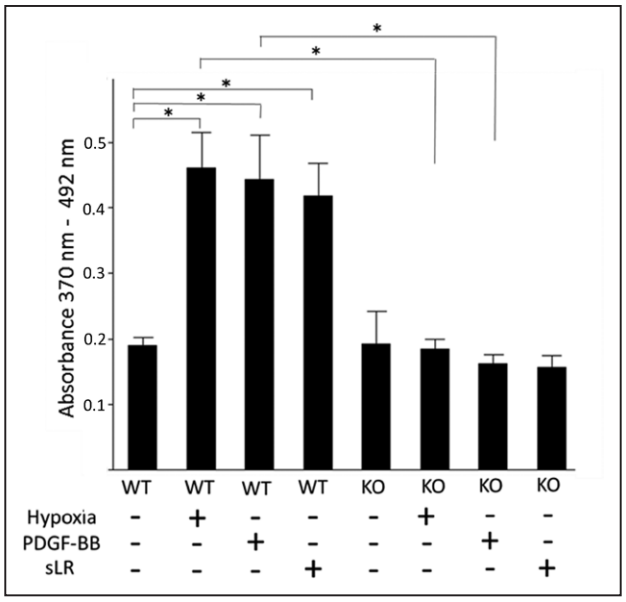

Figure 4. Role of LR11 (low-density lipoprotein receptor with 11 binding repeats) in pulmonary smooth muscle cell proliferation. Comparison of proliferation of pulmonary smooth muscle cell (SMC) from LR11$^{-/-}$ and wild-type (WT) mice. Exogenous sLR11 (soluble LR11; 10 ng/mL) induced proliferation in WT (0.19±0.01 vs 0.42±0.02 nm; P<0.05). Rate of proliferation is reduced in SMCs from LR11$^{-/-}$, compared with those from WT mice incubated with exogenous platelet-derived growth factor-BB (20 ng/mL; 0.16±0.01 vs. 0.45±0.07 nm; P<0.05) or under hypoxia (1% O_2 and 5% CO_2) for 48 h (0.18±0.02 vs 0.45±0.05 nm; P<0.05); n=4 each; *P<0.05. Statistical significance was determined with Tukey honestly significant difference. KO indicates knockout.

hypertension and LR11 expression increased dependently on HIF-1α under hypoxia. Serum sLR11 levels were also higher in patients with, than without, PAH and such increases were associated with mean pulmonary arterial pressure or pulmonary vascular resistance. This finding indicates that sLR11 serves as a marker of PASMC proliferation in patients with PAH.

Table 1. Comparison Between Patients With and Without PAH

	PH(–), n=9	PH(+), n=11	*P*
Age, y	67.1±9.9 (n=9)	54.0±20.8 (n=11)	0.084
No. of females, %	8 (88.9%)	9 (81.8%)	1.000
WHO FC (I/II/III/IV)	0/9/0/0	0/10/1/0	1.000
6MWD, min	376.3±95.9 (n=3)	328.6±135.6 (n=8)	0.595
LR11, ng/mL	9.2±4.1 (n=9)	14.2±4.5 (n=11)	0.019
BNP, pg/mL	169.9±187.4, n=9	84.3±102.8, n=11	0.210
UA, mg/dL	5.2±1.6 (n=9)	5.7±0.9 (n=11)	0.347
mRAP, mm Hg	4.8±3.1 (n=9)	5.1±3.2 (n=10)	0.825
mPAP, mm Hg	14.0±2.1 (n=9)	32.0±7.4 (n=11)	<0.001
PVR, dyne·s·cm^{-5}	148.8±53.4 (n=9)	427.6 ± 203.1 (n=11)	<0.001
CI, L/min/m^2	2.8±0.7 (n=9)	2.9 ± 0.6 (n=11)	0.658

6MWD indicates 6-min walking distance; BNP, brain natriuretic peptide; CI, cardiac index; LR11, low-density lipoprotein receptor with 11 binding repeats; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; PVR, pulmonary vascular resistance; UA, uric acid; and WHO FC, World Health Organization functional class.

LR11 Deletion Prevents Pulmonary Vascular Remodeling via HIF-1α-Mediated Signals

We investigated whether hypoxia induces HIF-1α–dependent LR11 expression in vivo and in vitro to determine the mechanisms of vascular remodeling in PH. We found that deleting LR11 prevented the development of pulmonary hypertension in a mouse model of PH. We previously reported that deleting LR11 in mice with atherosclerosis prevents vascular remodeling such as VSMC proliferation and migration.[17] The present findings showed that an LR11 deletion suppressed vascular thickening induced by hypoxia and that serum sLR11 levels were increased under hypoxia in wild-type mouse models of PH. We also investigated the role of LR11 in the proliferation of PASMC using LR11-deficient PASMC. The proliferation of PASMC from LR11$^{-/-}$ mice under hypoxia was reduced, whereas that from WT mice was increased by exogenous sLR11 protein, suggesting that LR11 deletion regulates SMC proliferation. HIF-1α plays a critical role in the pathological status of PH in these models of hypoxia.[18] We previously reported that HIF-1α binds to the proximal 144-bp LR11 promoter in a region where a potential HIF-1–binding site can be induced by hypoxia.[19] The present study found that HIF-1α knockdown decreased LR11 expression under hypoxia, indicating that HIF-1α plays a key role in LR11 expression under such conditions.

Soluble LR11 as a Biomarker of Proliferative SMC in Patients With PAH

The early phases of PAH are considered to be histologically nonspecific, with medial pulmonary arterial and adventitial thickening and the appearance of muscle in the walls of normally nonmuscular arteries being the only abnormalities.[20] Many current screening modalities depend on detecting an increase in pulmonary arterial pressure, and thus the early stages of pulmonary vascular disease are likely to be overlooked.[21] One mechanism of vascular remodeling is VSMC proliferation.[20] We previously reported that sLR11 is a biomarker of VSMC proliferation in atherosclerosis.[11,13,15] That prospective study of a small cohort of naive patients uncovered a relationship between sLR11 and mean pulmonary arterial pressure or pulmonary vascular resistance. Levels of sLR11 were significantly increased in patients with, rather than without, PAH. Serum sLR11 levels were 7.8±1.6 ng/mL in 56 healthy volunteers (data not shown). These levels significantly differed between healthy volunteers and patients with PAH but not between healthy volunteers and patients without PAH. Therefore, sLR11 might serve as a biomarker indicating SMC proliferation in the process of pulmonary arterial remodeling. Although several serological markers of PAH have been investigated, only BNP and N-terminal pro b-type natriuretic peptide have been included as prognostic parameters in treatment guidelines to date.[6] The proteins BNP/N-terminal pro b-type natriuretic peptide are associated with pulmonary hemodynamics and right ventricular dysfunction in PH, and BNP levels within World Health Organization functional class II do not increase.[22–24] We previously reported that sLR11 is produced by immature, but not by mature, SMC, in atherosclerotic arteries and that LR11 mRNA expression is high in human CD34$^+$ and

Table 2. Pearson Correlation Coefficient Between sLR11 Levels and Variables

n=20	n	Pearson Correlation Coefficient	*P*
Age, y	20	−0.029	0.902
Women, n	20	0.075	0.754
WHO FC (I/II/III/IV)	20	0.317	0.173
BNP, pg/mL	20	0.017	0.945
UA, mg/dL	20	0.054	0.821
mRAP, mm Hg	19	0.137	0.575
mPAP, mm Hg	20	0.633	0.003
PVR (dyne·sec·cm^{-5})	20	0.580	0.007
CI, L/min/m^2	20	−0.093	0.697

BNP indicates brain natriuretic peptide; CI, cardiac index; LR11, low-density lipoprotein receptor with 11 binding repeats; mPAP, mean pulmonary arterial pressure; mRAP, mean right atrial pressure; PVR, pulmonary vascular resistance; UA, uric acid; and WHO FC, World Health Organization functional class.

CD38$^-$ immature hematopoietic progenitors.[17,19,25] Considering the difficulties involved in trying to identify the major source of cells that regulate circulating sLR11 levels, we analyzed LR11 expression in rat monocrotaline and mouse hypoxia models. Levels of LR11 protein increased in both models, indicating that LR11 expression increased in the present models of PAH under hypoxia and under treatment with monocrotaline (data not shown). The findings suggested that LR11 expression is increased in association with the SMC proliferation during the progress of PAH, and not only under hypoxia. Although we found that sLR11 alone induced the proliferation of cultured PASMC, endothelial cells and immature cells such as hematopoietic stem and progenitor cells might also regulate serum sLR11 levels in pulmonary hypertension.[19,26] We previously showed that sLR11 could be a useful biomarker of conditions such as atherosclerosis,[17] large B-cell lymphoma,[27] non-Hodgkin lymphoma,[28] follicular lymphoma,[29] diabetic retinopathy,[30] and acute coronary syndrome.[31] Overall, sLR11 might serve as a potentially noninvasive and objective parameter of responses to therapy, although further investigation using other models of PH is essential to define how much circulating sLR11 levels reflect the pathological conditions of PASMC in patients.

We recently found that plasma cyclophilin A could serve as a marker of VSMC proliferation in PH and that plasma cyclophilin A levels increase in patients with PH according to the severity of pulmonary vascular resistance.[32] The PAH in the present study was mainly within World Health Organization functional class II, and therefore, we think that sLR11 will be useful to detect PAH. Further investigation is needed to confirm whether sLR11 can serve as a biomarker of the effects of drugs on PAH.

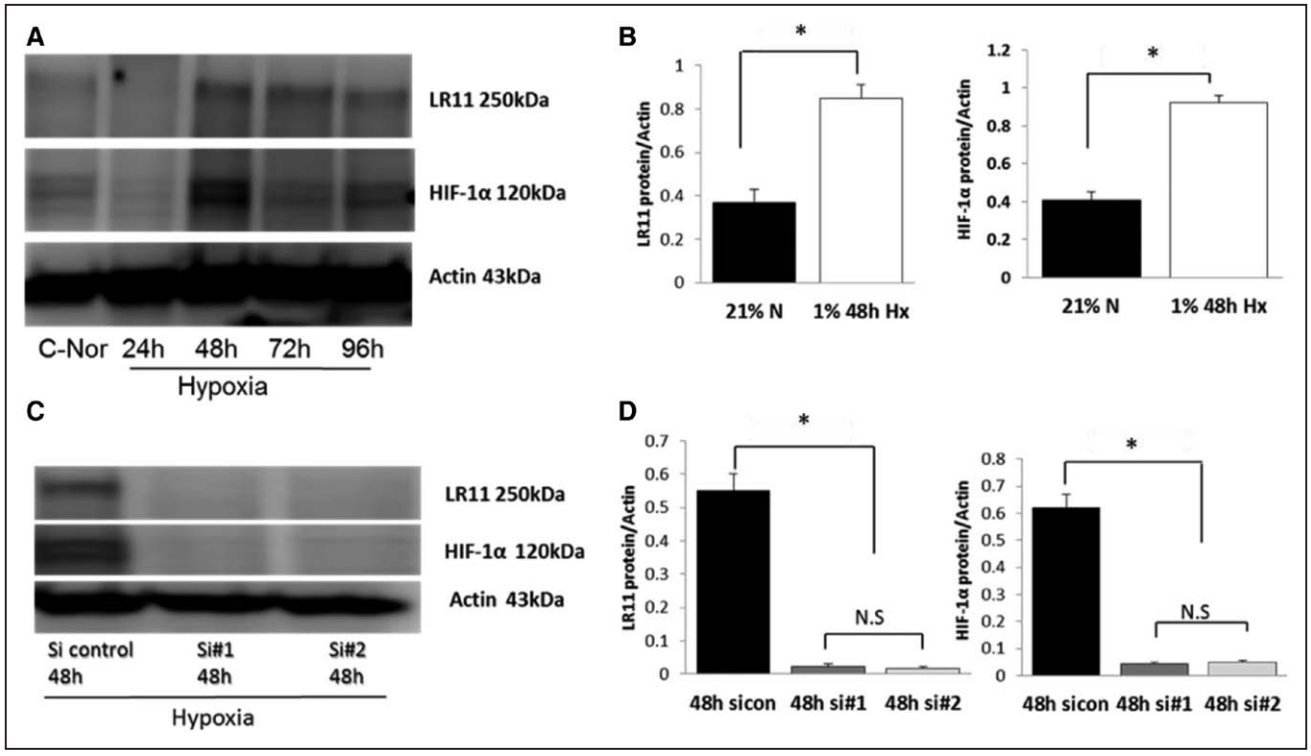

Figure 5. Expression of LR11 (low-density lipoprotein receptor with 11 binding repeats) and HIF-1α in human arterial SMC under hypoxia. **A**, Levels of LR11 and hypoxia-inducible factor-1α (HIF-1α) protein analyzed by Western blotting after 24, 48, 72, and 96 h under hypoxic conditions (1% O_2 and 5% CO_2). **B**, Levels of LR11 and HIF-1α protein expression peaked at 48 h under hypoxia compared with normoxia (0.9±0.02 vs. 0.31±0.03; * *P*<0.05 and 1.17±0.04 vs. 0.42±0.07; **P*<0.05, respectively). Statistical significance was determined with unpaired Student *t* test. **C**, HIF-1α expression in human pulmonary arterial smooth muscle cell was knocked down using small interfering RNA (siRNA) and then LR11 and HIF-1α protein expression was compared in cells incubated with 2 specific siRNA and 1 nonspecific control. **D**, Levels of LR11 and HIF-1α protein expression decreased compared with nonspecific controls (0.54±0.05 vs 0.02±0.01, 0.02±0.01; **P*<0.05 and 0.61±0.05 vs 0.04±0.01, 0.05±0.01; **P*<0.05, respectively). Statistical significance was determined with Tukey honestly significant difference.

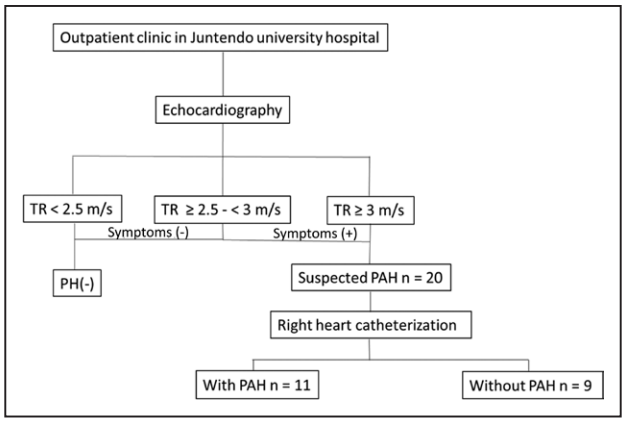

Figure 6. Flow of 20 consecutive prospectively enrolled patients suspected pulmonary hypertension through the study. Inclusion criteria for suspected pulmonary arterial hypertension (PAH) comprised echocardiographic and tricuspid regurgitation (TR) velocity ≥3.0 or 2.5 m/s with symptoms such as dyspnea. Eleven patients were diagnosed with PAH defined as mean pulmonary arterial pressure ≥25 mm Hg and pulmonary capillary wedge pressure ≤15 mm Hg.

The present study found that sLR11 induced the proliferation of PASMC, which could be a therapeutic target of pulmonary hypertension. In this context, the effects of statins on sLR11-mediated PASMC proliferation should be determined, because statins might be effective against PAH[33] and inhibit the LR11-induced migration of intimal SMC.[34]

In conclusion, this description of sLR11 serves as a biomarker of VSMC proliferation in PH. A deletion of LR11 prevents the development of pulmonary hypertension, indicating that LR11 is an important factor in the progression of this disease.

Acknowledgments

We are grateful to Dr J. Takagi (Laboratory of Protein Synthesis and Expression, Institute for Protein Research, Osaka University) for providing recombinant LR11 Vps10 domain protein.

Sources of Funding

This study was supported by JSPS KAKENHI grant number 24591069, 15K09127.

Disclosures

None.

References

1. D'Alonzo GE, Barst RJ, Ayres SM, Bergofsky EH, Brundage BH, Detre KM, Fishman AP, Goldring RM, Groves BM, Kernis JT. Survival in patients with primary pulmonary hypertension. Results from a national prospective registry. *Ann Intern Med*. 1991;115:343–349.
2. Galiè N, Rubin Lj, Hoeper M, Jansa P, Al-Hiti H, Meyer G, Chiossi E, Kusic-Pajic A, Simonneau G. Treatment of patients with mildly symptomatic pulmonary arterial hypertension with bosentan (EARLY study): a double-blind, randomised controlled trial. *Lancet*. 2008;371:2093–2100. doi: 10.1016/S0140-6736(08)60919-8.
3. Rubin LJ, Badesch DB, Fleming TR, Galiè N, Simonneau G, Ghofrani HA, Oakes M, Layton G, Serdarevic-Pehar M, McLaughlin VV, Barst RJ; SUPER-2 Study Group. Long-term treatment with sildenafil citrate in pulmonary arterial hypertension: the SUPER-2 study. *Chest*. 2011;140:1274–1283. doi: 10.1378/chest.10-0969.
4. Rubin LJ, Galiè N, Grimminger F, Grünig E, Humbert M, Jing ZC, Keogh A, Langleben D, Fritsch A, Menezes F, Davie N, Ghofrani HA. Riociguat for the treatment of pulmonary arterial hypertension: a long-term extension study (PATENT-2). *Eur Respir J*. 2015;45:1303–1313. doi: 10.1183/09031936.00090614.
5. Pulido T, Adzerikho I, Channick RN, et al; SERAPHIN Investigators. Macitentan and morbidity and mortality in pulmonary arterial hypertension. *N Engl J Med*. 2013;369:809–818. doi: 10.1056/NEJMoa1213917.
6. Galiè N, Hoeper MM, Humbert M, et al; ESC Committee for Practice Guidelines (CPG). Guidelines for the diagnosis and treatment of pulmonary hypertension: the Task Force for the Diagnosis and Treatment of Pulmonary Hypertension of the European Society of Cardiology (ESC) and the European Respiratory Society (ERS), endorsed by the International Society of Heart and Lung Transplantation (ISHLT). *Eur Heart J*. 2009;30:2493–2537. doi: 10.1093/eurheartj/ehp297.
7. Guignabert C, Tu L, Girerd B, Ricard N, Huertas A, Montani D, Humbert M. New molecular targets of pulmonary vascular remodeling in pulmonary arterial hypertension: importance of endothelial communication. *Chest*. 2015;147:529–537. doi: 10.1378/chest.14-0862.
8. Heath D, Smith P, Gosney J, Mulcahy D, Fox K, Yacoub M, Harris P. The pathology of the early and late stages of primary pulmonary hypertension. *Br Heart J*. 1987;58:204–213.
9. Hassoun PM, Mouthon L, Barberà JA, et al. Inflammation, growth factors, and pulmonary vascular remodeling. *J Am Coll Cardiol*. 2009;54(suppl 1):S10–S19. doi: 10.1016/j.jacc.2009.04.006.
10. Yamazaki H, Bujo H, Kusunoki J, Seimiya K, Kanaki T, Morisaki N, Schneider WJ, Saito Y. Elements of neural adhesion molecules and a yeast vacuolar protein sorting receptor are present in a novel mammalian low density lipoprotein receptor family member. *J Biol Chem*. 1996;271:24761–24768.
11. Kanaki T, Bujo H, Hirayama S, Ishii I, Morisaki N, Schneider WJ, Saito Y. Expression of LR11, a mosaic LDL receptor family member, is markedly increased in atherosclerotic lesions. *Arterioscler Thromb Vasc Biol*. 1999;19:2687–2695.
12. Zhu Y, Bujo H, Yamazaki H, Hirayama S, Kanaki T, Takahashi K, Shibasaki M, Schneider WJ, Saito Y. Enhanced expression of the LDL receptor family member LR11 increases migration of smooth muscle cells *in vitro*. *Circulation*. 2002;105:1830–1836.
13. Ohwaki K, Bujo H, Jiang M, Yamazaki H, Schneider WJ, Saito Y. A secreted soluble form of LR11, specifically expressed in intimal smooth muscle cells, accelerates formation of lipid-laden macrophages. *Arterioscler Thromb Vasc Biol*. 2007;27:1050–1056. doi: 10.1161/ATVBAHA.106.137091.
14. Zhu Y, Bujo H, Yamazaki H, Ohwaki K, Jiang M, Hirayama S, Kanaki T, Shibasaki M, Takahashi K, Schneider WJ, Saito Y. LR11, an LDL receptor gene family member, is a novel regulator of smooth muscle cell migration. *Circ Res*. 2004;94:752–758. doi: 10.1161/01.RES.0000120862.79154.0F.
15. Ogita M, Miyauchi K, Jiang M, Kasai T, Tsuboi S, Naito R, Konishi H, Dohi T, Yokoyama T, Okazaki S, Shimada K, Bujo H, Daida H. Circulating soluble LR11, a novel marker of smooth muscle cell proliferation, is enhanced after coronary stenting in response to vascular injury. *Atherosclerosis*. 2014;237:374–378. doi: 10.1016/j.atherosclerosis.2014.08.044.
16. Frank DB, Abtahi A, Yamaguchi DJ, Manning S, Shyr Y, Pozzi A, Baldwin HS, Johnson JE, de Caestecker MP. Bone morphogenetic protein 4 promotes pulmonary vascular remodeling in hypoxic pulmonary hypertension. *Circ Res*. 2005;97:496–504. doi: 10.1161/01.RES.0000181152.65534.07.
17. Jiang M, Bujo H, Ohwaki K, Unoki H, Yamazaki H, Kanaki T, Shibasaki M, Azuma K, Harigaya K, Schneider WJ, Saito Y. Ang II-stimulated migration of vascular smooth muscle cells is dependent on LR11 in mice. *J Clin Invest*. 2008;118:2733–2746. doi: 10.1172/JCI32381.
18. Yu AY, Shimoda LA, Iyer NV, Huso DL, Sun X, McWilliams R, Beaty T, Sham JS, Wiener CM, Sylvester JT, Semenza GL. Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. *J Clin Invest*. 1999;103:691–696. doi: 10.1172/JCI5912.
19. Nishii K, Nakaseko C, Jiang M, Shimizu N, Takeuchi M, Schneider WJ, Bujo H. The soluble form of LR11 protein is a regulator of hypoxia-induced, urokinase-type plasminogen activator receptor (uPAR)-mediated adhesion of immature hematological cells. *J Biol Chem*. 2013;288:11877–11886. doi: 10.1074/jbc.M112.442491.
20. Tuder RM, Marecki JC, Richter A, Fijalkowska I, Flores S. Pathology of pulmonary hypertension. *Clin Chest Med*. 2007;28:23–42, vii. doi: 10.1016/j.ccm.2006.11.010.

21. Lau EM, Manes A, Celermajer DS, Galiè N. Early detection of pulmonary vascular disease in pulmonary arterial hypertension: time to move forward. *Eur Heart J*. 2011;32:2489–2498. doi: 10.1093/eurheartj/ehr160.
22. Nagaya N, Nishikimi T, Okano Y, Uematsu M, Satoh T, Kyotani S, Kuribayashi S, Hamada S, Kakishita M, Nakanishi N, Takamiya M, Kunieda T, Matsuo H, Kangawa K. Plasma brain natriuretic peptide levels increase in proportion to the extent of right ventricular dysfunction in pulmonary hypertension. *J Am Coll Cardiol*. 1998;31:202–208.
23. Leuchte HH, Holzapfel M, Baumgartner RA, Ding I, Neurohr C, Vogeser M, Kolbe T, Schwaiblmair M, Behr J. Clinical significance of brain natriuretic peptide in primary pulmonary hypertension. *J Am Coll Cardiol*. 2004;43:764–770. doi: 10.1016/j.jacc.2003.09.051.
24. Cracowski JL, Yaici A, Sitbon O, et al. [Biomarkers as prognostic factors in pulmonary arterial hypertension. Rationale and study design]. *Rev Mal Respir*. 2004;21(6 pt 1):1137–1143.
25. Zhang X, Dormady SP, Basch RS. Identification of four human cDNAs that are differentially expressed by early hematopoietic progenitors. *Exp Hematol*. 2000;28:1286–1296.
26. Ting HJ, Stice JP, Schaff UY, Hui DY, Rutledge JC, Knowlton AA, Passerini AG, Simon SI. Triglyceride-rich lipoproteins prime aortic endothelium for an enhanced inflammatory response to tumor necrosis factor-alpha. *Circ Res*. 2007;100:381–390. doi: 10.1161/01.RES.0000258023.76515.a3.
27. Kawaguchi T, Ohwada C, Takeuchi M, et al. Potential utility of serum soluble LR11 as a diagnostic biomarker for intravascular large B-cell lymphoma. *Leuk Lymphoma*. 2014;55:2391–2394. doi: 10.3109/10428194.2014.880430.
28. Fujimura K, Ebinuma H, Fukamachi I, Ohwada C, Kawaguchi T, Shimizu N, Takeuchi M, Sakaida E, Jiang M, Nakaseko C, Bujo H. Circulating LR11 is a novel soluble-receptor marker for early-stage clinical conditions in patients with non-Hodgkin's lymphoma. *Clin Chim Acta*. 2014;430:48–54. doi: 10.1016/j.cca.2013.12.039.
29. Kawaguchi T, Ohwada C, Takeuchi M, et al. LR11: a novel biomarker identified in follicular lymphoma. *Br J Haematol*. 2013;163:277–280. doi: 10.1111/bjh.12467.
30. Takahashi M, Bujo H, Shiba T, Jiang M, Maeno T, Shirai K. Enhanced circulating soluble LR11 in patients with diabetic retinopathy. *Am J Ophthalmol*. 2012;154:187–192. doi: 10.1016/j.ajo.2012.01.035.
31. Ogita M, Miyauchi K, Dohi T, Tsuboi S, Miyazaki T, Yokoyama T, Yokoyama K, Shimada K, Kurata T, Jiang M, Bujo H, Daida H. Increased circulating soluble LR11 in patients with acute coronary syndrome. *Clin Chim Acta*. 2013;415:191–194. doi: 10.1016/j.cca.2012.10.047.
32. Satoh K, Satoh T, Kikuchi N, et al. Basigin mediates pulmonary hypertension by promoting inflammation and vascular smooth muscle cell proliferation. *Circ Res*. 2014;115:738–750. doi: 10.1161/CIRCRESAHA.115.304563.
33. Katsiki N, Wierzbicki AS, Mikhailidis DP. Pulmonary arterial hypertension and statins: an update. *Curr Opin Cardiol*. 2011;26:322–326. doi: 10.1097/HCO.0b013e32834659bf.
34. Bujo H, Saito Y. Modulation of smooth muscle cell migration by members of the low-density lipoprotein receptor family. *Arterioscler Thromb Vasc Biol*. 2006;26:1246–1252. doi: 10.1161/01.ATV.0000219692.78477.17.

Highlights

- Deleting LR11 (low-density lipoprotein receptor with 11 binding repeats) did not result in pulmonary hypertension and LR11 expression increased dependently on hypoxia-inducible factor-1α under hypoxia.
- LR11 is a potential key regulator of smooth muscle cell proliferation under hypoxia.
- Serum soluble LR11 levels in patients with pulmonary arterial hypertension are increased and serum soluble LR11 was associated with mean pulmonary arterial pressure or pulmonary vascular resistance. Soluble LR11 might serve as a biomarker of pulmonary arterial remodeling.

Arteriosclerosis, Thrombosis, and Vascular Biology

JOURNAL OF THE AMERICAN HEART ASSOCIATION

Deletion of LR11 Attenuates Hypoxia-Induced Pulmonary Arterial Smooth Muscle Cell Proliferation With Medial Thickening in Mice

Le Jiang, Hakuoh Konishi, Fariz Nurwidya, Kimio Satoh, Fumiyuki Takahashi, Hiroyuki Ebinuma, Kengo Fujimura, Kiyoshi Takasu, Meizi Jiang, Hiroaki Shimokawa, Hideaki Bujo and Hiroyuki Daida

Arterioscler Thromb Vasc Biol. 2016;36:1972-1979; originally published online August 4, 2016;
doi: 10.1161/ATVBAHA.116.307900
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 1079-5642. Online ISSN: 1524-4636