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Deletion of LR11 attenuates hypoxia-induced pulmonary arterial smooth muscle cell proliferation with medial thickening in mice

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Running title: LR11 and pulmonary hypertension

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Objective

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

Approach and Results

The role of LR11 in PAH was investigated using mouse and cell models of induced hypoxia. The expression LR11 and of HIF-1α was significantly increased in lung tissues from C57Bl/6 mice after three weeks of exposure to hypoxia compared with normoxia. Serum sLR11 levels also were 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 PDGF-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 HIF-1α was increased in cultured SMC under hypoxic conditions and HIF-1α knockdown almost abolished the induction of LR11. Serum sLR11 levels were significantly higher in patients with, rather than without, PAH. 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 PAH.

Abbreviations

HIF-1α: hypoxia-inducible factor-1 alpha
LR11: low-density lipoprotein receptor with 11 binding repeats
mPAP: mean pulmonary arterial pressure
PAH: pulmonary arterial hypertension
PASMC: pulmonary arterial smooth muscle cells
PVR: pulmonary vascular resistance
RVSP: right ventricular systolic pressure
Si, small interfering
SMC, smooth muscle cells
TR, tricuspid regurgitation
VSMC: vascular smooth muscle cells

Introduction

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(ET-1) receptor antagonist, phosphodiesterase-5 (PDE5) 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/NT-proBNP has served as a sensitive marker in this capacity[6]. Thus, novel circulating molecules based on a pathophysiology that differs from that of BNP/NT-proBNP are required to increase the ability to detect initial disease progression and improve individual treatment strategies. One of candidates for such markers may be a circulating molecule which represents the proliferation of smooth muscle cells (SMCs) in the progress of medial thickening. These cells proliferate abnormally under crosstalk with dysfunctional endothelial cells (ECs) and other components of the pulmonary vascular wall including myofibroblasts, pericytes and circulating immune cells and thus, a complicated mechanism underlies the SMC proliferation, one of key features in PAH pathogenesis[7]. These cells proliferate abnormally under crosstalk with dysfunctional endothelial cells (ECs) and other components of the pulmonary vascular wall including myofibroblasts, pericytes and circulating immune cells. Therefore, the mechanism underlying SMC proliferation is complicated, and a key feature of PAH pathogenesis[7]. Vascular smooth muscle cells (VSMC) proliferate in the media of the pulmonary artery of patients with PAH[8] and several growth factors such as platelet-derived growth factor (PDGF) 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 (LDL) receptor[10] that is expressed in intimal smooth muscle cells 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 vs. 0.53 ± 0.04 $p < 0.05$; Figure 1A and D), LR11 expression (0.25 ± 0.03 vs. 0.65 ± 0.04; $p < 0.05$; Figure 1A and B) 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.

LR11 (-/-) mice are resistant to induction of pulmonary hypertension

We investigated the role of LR11 in the development of PAH in LR11 (-/-) model 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 D). The RVSP and ratio of RV/LV+IVS were significantly lower in LR11 (-/-) than in WT mice. (28.3 ± 2.2 vs. 33.3 ± 2.2 mmHg, $p< 0.05$ and 30.9% ± 2.2% vs. 36.4% ± 2.4%, $p < 0.05$, respectively; Figure 2A, B and C). 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% vs. 5.7% ± 0.4%, $p < 0.05$; Figure 3A and B). The ratios of arteries categorized as muscular, partly muscular and non-muscular 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 non-muscular arteries in LR11 (-/-) mice were significantly increased compared with those in WT mice under hypoxia ($n = 4$, $^*P < 0.05$; C). 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 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 PDGF-BB (20 ng/mL) were significantly decreased in PASMC isolated from LR11 (-/-), compared with those from wild-type mice (0.18 ± 0.02 vs. 0.45 ± 0.05 nm, $P < 0.05$ and 0.16 ± 0.01 vs. 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 vs. 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 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 vs. 0.31 ± 0.03, $p < 0.05$; Figure 5A and B). The transient increase in LR11 at this time point was similar to that of HIF-1α (0.92 ± 0.05 vs. 0.41 ± 0.04, $p < 0.05$; Figure 5A and B). Thus, we analyzed the effect of HIF1α-knockdown on LR11 expression after hypoxia. The sharp increase in LR11 expression at 48 h was almost completely abrogated in HIF1α-knockdown PASMC under hypoxia (Figure 5C and D). 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 (Figure6) and then compared serum sLR11 levels in patients with and without confirmed PAH whose age, sex and World Health Organization (WHO) functional class did not significantly differ (Table 1). Most patients had WHO functional class II PAH. Connective tissue diseases were associated with PAH in nine of 11 patients and two had portopulmonary hypertension (PoPH). 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 vs. 9.2 ± 4.1 ng/mL; P = 0.019), whereas levels of BNP and UA did not significantly differ (Table 1). Pearson's correlation coefficient analyses showed that the sLR11 levels positively correlated with mPAP (r = 0.633, P = 0.003) and PVR (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 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 mPAP or PVR. This finding indicates that sLR11 serves as a marker of PASMC proliferation in patients with PAH.

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 wild-type 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 non-muscular arteries being the only abnormalities[20]. Many current screening modalities depend on detecting an increase in PAP, 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 naïve patients uncovered a relationship between sLR11 and mPAP or PVR. 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 NT-proBNP have been included as prognostic parameters in treatment guidelines to date[6]. The proteins BNP/NT-proBNP are associated with pulmonary hemodynamics and right ventricular dysfunction in PH, and BNP levels within WHO functional class (FC) 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 $CD34^+$ $CD38^-$ 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 HSPC 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's 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 (CyPA) could serve as a marker of VSMC proliferation in PH and that plasma CyPA levels increase in patients with PH according to the severity of pulmonary vascular resistance[32]. The PAH in the present study was mainly within WHO FC II, and therefore, we believe 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.

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 is the first description of sLR11 serving 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.

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Disclosures

None to declare.

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Highlights

- Deleting LR11 did not result in pulmonary hypertension and LR11 expression increased dependently upon HIF-1α under hypoxia.
- LR11 is a potential key regulator of SMC proliferation under hypoxia.
- Serum sLR11 levels in patients with PAH are increased and serum sLR11 was associated with mPAP or PVR. Soluble LR11 might serve as a biomarker of pulmonary arterial remodeling.

Table 1. Comparison between patients with and without PAH.

	PH (-) n=9	PH (+) n=11	P
Age (years)	67.1 ± 9.9 (n = 9)	54.0 ± 20.8 (n = 11)	0.084
Number 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 (m)	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 (mmHg)	4.8 ± 3.1 (n = 9)	5.1 ± 3.2 (n = 10)	0.825
mPAP (mmHg)	14.0 ± 2.1 (n = 9)	32.0 ± 7.4 (n = 11)	< 0.001
PVR (dyne·sec·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

Abbreviations: 6MWD, 6-minute 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; WHO FC, World Health Organization functional class.

Table 2. Pearson's correlation coefficient between sLR11 levels and variables.

n = 20	n	Pearson's correlation coefficient	P
Age (years)	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 (mmHg)	19	0.137	0.575
mPAP (mmHg)	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

Abbreviations: 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, WHO FC, World Health Organization functional class.

Figure 1. **Western blots of LR11 and HIF-1α protein expression in lungs of mice exposed to normoxia or hypoxia.**

A and B. LR11 protein expression significantly increased in lungs of WT mice exposed to hypoxia compared with normoxia.
A and D. Expression of HIF-1α protein significantly increased in wild type (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 increased in WT mice after three weeks 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's t-test.

Figure 2. Effects of LR11 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 three weeks.
B. Pulmonary hypertension did not develop in LR11 (-/-) mice compared with WT mice. (28.3 ± 2.2 vs. 33.3 ± 2.2 mmHg,*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 weeks. 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 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 weeks. Lung sections were stained with hematoxylin eosin

and Elastic van Gieson. Magnification, ×40; scale 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 weeks (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 three arteries categorized as muscular, partly muscular and non-muscular, 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 non-muscular arteries were significantly increased in LR11 (-/-) compared with WT mice under hypoxia (n = 4 each, *P < 0.05).
D. Number of CD45-positive cells in distal pulmonary artery adventitia was significantly reduced in LR11-/- compared with WT mice after exposure to hypoxia. Number of CD45-positive 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, Elastica van Gieson; HE, hematoxylin eosin.

Figure 4. Role of LR11 in pulmonary smooth muscle cell proliferation.

Comparison of proliferation of pulmonary SMC from LR11 (-/-) and wild type (WT) mice. Exogenous sLR11 (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 PDGF-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 hours (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.

Figure 5. Expression of LR11 and HIF-1α in human arterial SMC under hypoxia.

A. Levels of LR11 and HIF-1α protein analyzed by Western blotting after 24, 48, 72 and 96 hours under hypoxic conditions (1% O_2 and 5% CO_2).
B. Levels of LR11 and HIF-1α protein expression peaked at 48 hours 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's t-test.
C. HIF-1α expression in human PASMC was knocked down using small interfering RNA (siRNA) and then LR11 and HIF-1α protein expression was compared in cells incubated with two specific siRNA and one non-specific control.
D. Levels of LR11 and HIF-1α protein expression decreased compared with non-specific 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 PAH comprised echocardiographic and TR velocity ≥ 3.0 or 2.5 m/s with symptoms such as dyspnea. Eleven patients were diagnosed with PAH defined as mean PAP ≥ 25 mmHg and PCWP ≤ 15 mmHg.
PAH, pulmonary arterial hypertension; PAP, pulmonary arterial pressure; PCWP, pulmonary capillary wedge pressure; TR, tricuspid regurgitation.

Materials and Methods

Animal models

LR11(-/-)[1] and control wild-type (C57Bl/6) mice aged 8 – 10 weeks (n = 8 per group) (Sankyo Labo Corporation Inc. Tokyo, Japan) were exposed to chronic hypoxia (CH; FIO_2 10%) or room air (Control) for three weeks as described[2] and had access to standard chow and water *ad libitum*.

Measurement of right ventricular pressure

Right ventricular systolic pressure (RVSP) was measured in mice lightly anesthetized with isoflurane using a Univentor 400 anesthesia unit (Univentor Ltd., Zejtun, Malta) The right internal jugular veins of the mice were surgically exposed and cannulated with a 1.4-F microtip pressure transducer (Millar Instruments, Houston TX, USA) before sacrifice. The transducer was advanced into the right ventricle, and right ventricular pressure was continuously monitored for 10 minutes. Heart rates (480 – 550 beats/min were deemed acceptable) and pressure waveforms were monitored to ensure the validity of the pressure measurements. Data were analyzed using PowerLab software (AD Instruments, Denver, CO, USA).

Measurement of right ventricular hypertrophy

The mice were sacrificed with isoflurane after measuring hemodynamic parameters and then the hearts were removed and placed in formalin for 24 hours. The free wall of the right ventricle (RV) was then carefully dissected from the left ventricle (LV) and septum (S) and all parts were individually weighed to calculate the ratio of RV/LV+S weight as

an index of right ventricular hypertrophy.

Histological evaluation

The lungs were fixed via an intra-tracheal infusion of 4% phosphate-buffered formalin at a pressure of 23 cmH_2O and then the tissues were embedded in paraffin, sectioned at a thickness of 5 µm for light microscopy and visualized by staining with hematoxylin eosin and Elastica van Gieson. Briefly, pulmonary vascular remodeling was assessed by measuring the media thickness of vessels (diameter 50 – 100 µm) indexed to terminal bronchioles and as changes in the ratios (%) of muscular, partly muscular and non-muscular arteries. Pulmonary vessel wall thickness was analyzed in a blinded fashion using KS400 morphometry software (Carl Zeiss, Oberkochen, Germany) as described[3, 4]. The ratio of wall thickness is expressed as medial wall thickness (distance between the internal and external elastic lamina) divided by the diameter of the vessel (distance between the external elastic lamina) × 100 (%). At least 10 arteries from five lung sections per mouse were analyzed. Arteries with an external diameter of 20 - 70 µm were considered fully muscularized if a double elastic lamina was distinctly visible throughout the diameter of vessel cross sections. Arteries were considered partially muscularized if a double elastic lamina was distinctly visible for at least half of the vessel diameter. The ratio of vessels with double elastic lamina was calculated as the number of muscularized vessels per total number of vessels counted. The number of inflammatory cells under hypoxia was evaluated in an immunohistochemical comparison using the common antigen for leukocytes, anti-mouse CD45 antibody (BD Pharmingen #550539).Cells positive for CD45 were counted in the distal pulmonary artery adventitia (diameter 50 – 100 µm).

Western blot analysis

Protein concentrations were determined in peripheral lung tissues that were collected and homogenized immediately after the mice were sacrificed. Whole lung homogenates (45 μg protein/lane) were resolved by 7.5% gradient SDS-PAGE gel (Invitrogen) electrophoresis and then proteins were electro-blotted onto polyvinylidene fluoride (PVDF) or nitrocellulose membranes. Non-specific binding was blocked with 5% skim milk and then proteins were probed using primary LR11-specific (1:1000, A2-2-3) or mouse monoclonal anti-HIF1α (1:1000, BD Transduction Laboratories #610958) antibodies on a rocking platform for one hour at room temperature. Washed membranes were incubated with horseradish peroxidase–conjugated secondary antibody. The expression of proteins was normalized to that of β-actin or GAPDH. All proteins on blots were immunodetected using the SuperSignal chemiluminescence method (Pierce Biotechnology, Rockford, IL, USA), and relative immunoreactive levels of proteins were quantified using the ChemiDoc XRS imaging system with Quantity One software (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Mouse serum s LR11 levels

Mouse serum sLR11 levels were measured using a specific ELISA. Recombinant LR11 vps10 domain protein was provided by Dr**.** J. Takagi[5]. We detected sLR11 in mouse, rat and rabbit sera using a sandwich ELISA and monoclonal antibodies (mAb) 93222 and 93213 that were raised by immunizing LR11-/- mice with recombinant LR11 vps10 domain protein. In brief, mouse serum samples were diluted with sample buffer, reacted

with the capture mAb 93222 for 2 h and then incubated with biotinylated mAb 93213 for 1 h. LR11-antibody complexes were quantified by comparison with standard rabbit serum using horseradish peroxidase-conjugated streptavidin. Levels of sLR11 in rabbit serum were determined as described[6].

Harvesting mouse pulmonary artery VSMC and cell proliferation assays

Mouse pulmonary arterial VSMCs from groups of 10-week-old male mice cultured in DMEM containing 10% FBS at 37ºC under a humidified atmosphere of 5% CO_2 and 95% air as described[4] were used at passages 4 to 6. Mouse pulmonary arterial VSMC (70% - 80% confluence) were seeded into 96-well plates (5,000 cells/well) in DMEM containing 10% FBS. The VSMC were starved on the following day and then stimulated with either PDGF-BB (20 ng/mL) or sLR11 (10 ng/mL), or placed under hypoxia (1% O_2 for 48 hours). Thereafter, BrdU was added, the cells were incubated for two hours and then cell proliferation was assessed as the rate of BrdU incorporation determined using enzyme-linked immunosorbent assay (ELISA) kits (Roche, Mannheim, Germany).

Cell culture under hypoxia

Human PASMC from healthy individuals (Promocell GmbH, Heidelberg, Germany) were cultured under hypoxic conditions (1% O_2 and 5% CO_2) in an Oxygen Controller ProOx110 **(**Biospherix Ltd., Lacona, NY, USA) and then LR11 and HIF-1α expression was assessed in PASMC at 24, 48, 72 and 96 hours later by Western blotting.

RNA interference

We investigated how HIF-1α regulates LR11 expression in PAVSMC using custom-synthesized small interfering RNA (siRNA) targeting HIF1-α (Invitrogen,

Waltham, MA, USA) and a negative control (Invitrogen). Human PASMC were transfected with two specific siRNA and one non-specific control using Lipofectamine RNAiMAX (Invitrogen) as described[7]. The cells were detached and diluted in complete growth medium without antibiotics and seeded. Lipofectamine™ RNAiMAX and RNAi duplex were mixed with Opti-MEM®I (Gibco-BRL, Grand Island, NY, USA) and medium reduced serum. After adding complexes of RNAi duplex-Lipofectamine™ RNAiMAX (Life Technologies, Carlsbad, CA, USA), the cells were incubated for 48 hours at 37°C. The sequences of the siRNA against HIF-1α were as follows:

HIF1-α (1): 5′-AGUUAGUUCAAACUGAGUUAAUCCC-3′

HIF1-α (2): 5′-AUAUGAUUGUGUCUCCAGCGGCUGG -3′

Selection of patients

We prospectively enrolled 20 consecutive patients with suspected pulmonary arterial hypertension between May 2010 and April 2014 at Juntendo University Hospital. The criteria for suspected PAH comprised echocardiographic findings and a tricuspid regurgitation (TR) velocity ≥ 3.0 m/s, or ≥ 2.5 m/s accompanied by symptoms such as dyspnea or general fatigue (Figure 1). Patients were diagnosed with PAH if they had mean pulmonary arterial pressure (mPAP) ≥ 25 mmHg and pulmonary capillary wedge pressure (PCWP) ≤ 15 mmHg. Serum was separated from blood samples by centrifugation at 1000 × g for 10 min and stored at -80°C. Soluble LR11 was measured using a sandwich enzyme-linked immunosorbent assay (ELISA) with specific monoclonal antibodies directed against human LR11[6]. Other markers such as BNP and uric acid were determined using routine laboratory assays.

Statistical analysis

Data are expressed as means ± SD or ratios (%) and categorical data are expressed as numbers. All data were analyzed using JMP Statistical Discovery Software 10.0 (SAS Institute, Cary, NC, USA). Comparisons of means between 2 groups were performed by unpaired Student's t-test. Comparisons of means among multiple groups were performed by two-way analysis of variance (ANOVA), followed by Tukey's HSD (honestly significant difference) multiple comparisons. Categorical variables (presented as frequencies) were compared using the chi-square test. Associations between measured parameters were analyzed using Pearson's correlation coefficient. P-value less than 0.05 was considered to be statistically significant.

Ethics statement

All patients provided written informed consent to participate in the study, which was approved by the Human Ethics Review Committee of Juntendo University and conformed to the Declaration of Helsinki. All experimental animal studies proceeded in accordance with the protocols approved by the Juntendo University Animal Care and Use Committee.

1. 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.
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Johnson JE, de Caestecker MP. Bone morphogenetic protein 4 promotes pulmonary vascular remodeling in hypoxic pulmonary hypertension. Circ Res. 2005;97:496-504.

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4. 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.
5. Nakata Z, Nagae M, Yasui N, Bujo H, Nogi T, Takagi J. Crystallization and preliminary crystallographic analysis of human LR11 Vps10p domain. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2011;67:129-132.
6. 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.
7. Murakami A, Takahashi F, Nurwidya et al. Hypoxia increases gefitinib-resistant lung cancer stem cells through the activation of insulin-like growth factor 1 receptor. PloS one. 2014;9:e86459.

Figure 1

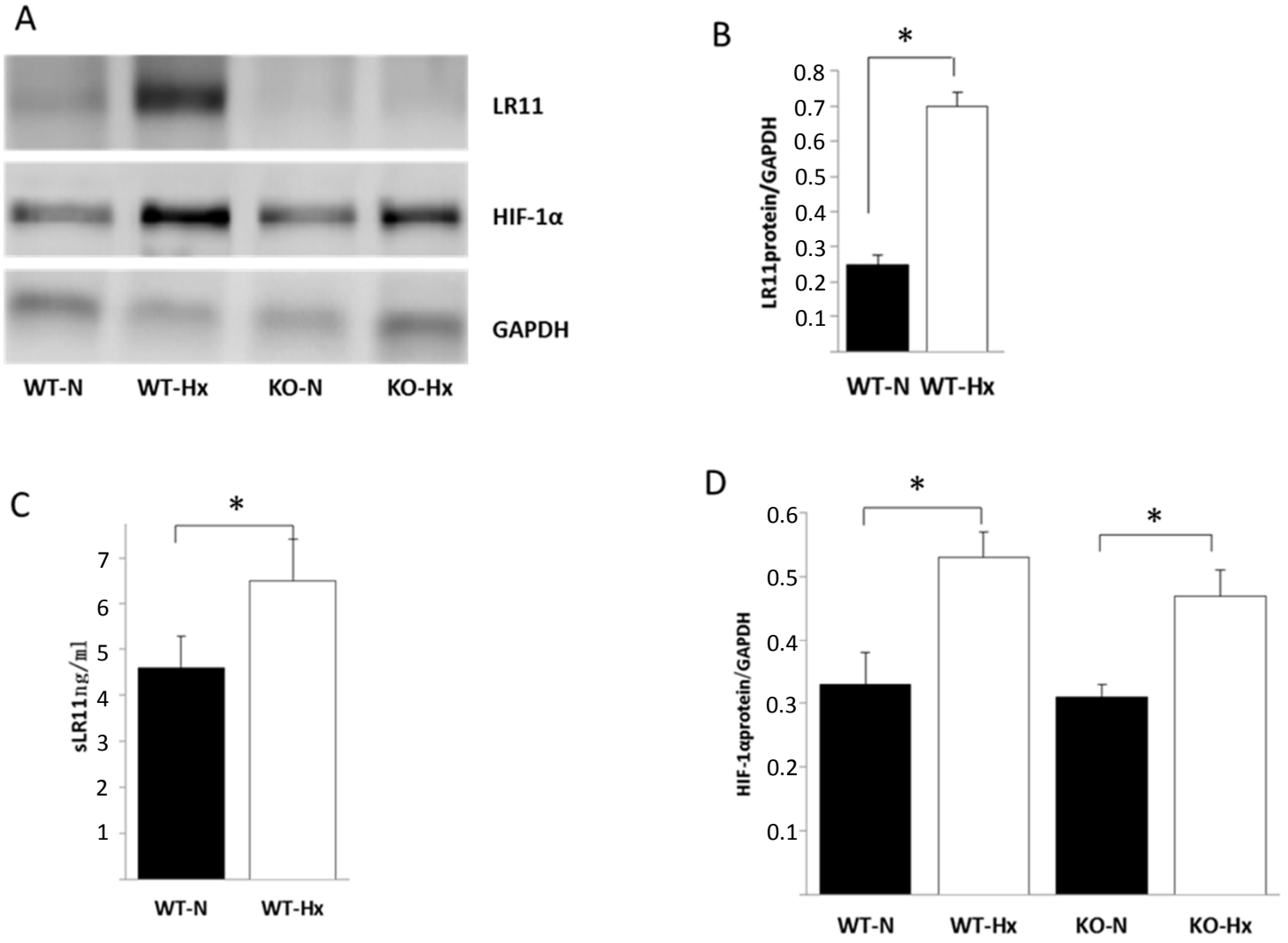

A
LR11
HIF-1α
GAPDH
WT-N
WT-Hx
KO-N
KO-Hx
B
*
LR11protein/GAPDH
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
WT-N
WT-Hx
C
*
sLR11ng/ml
7
6
5
4
3
2
1
WT-N
WT-Hx
D
*
*
HIF-1αprotein/GAPDH
0.6
0.5
0.4
0.3
0.2
0.1
WT-N
WT-Hx
KO-N
KO-Hx

Figure 2

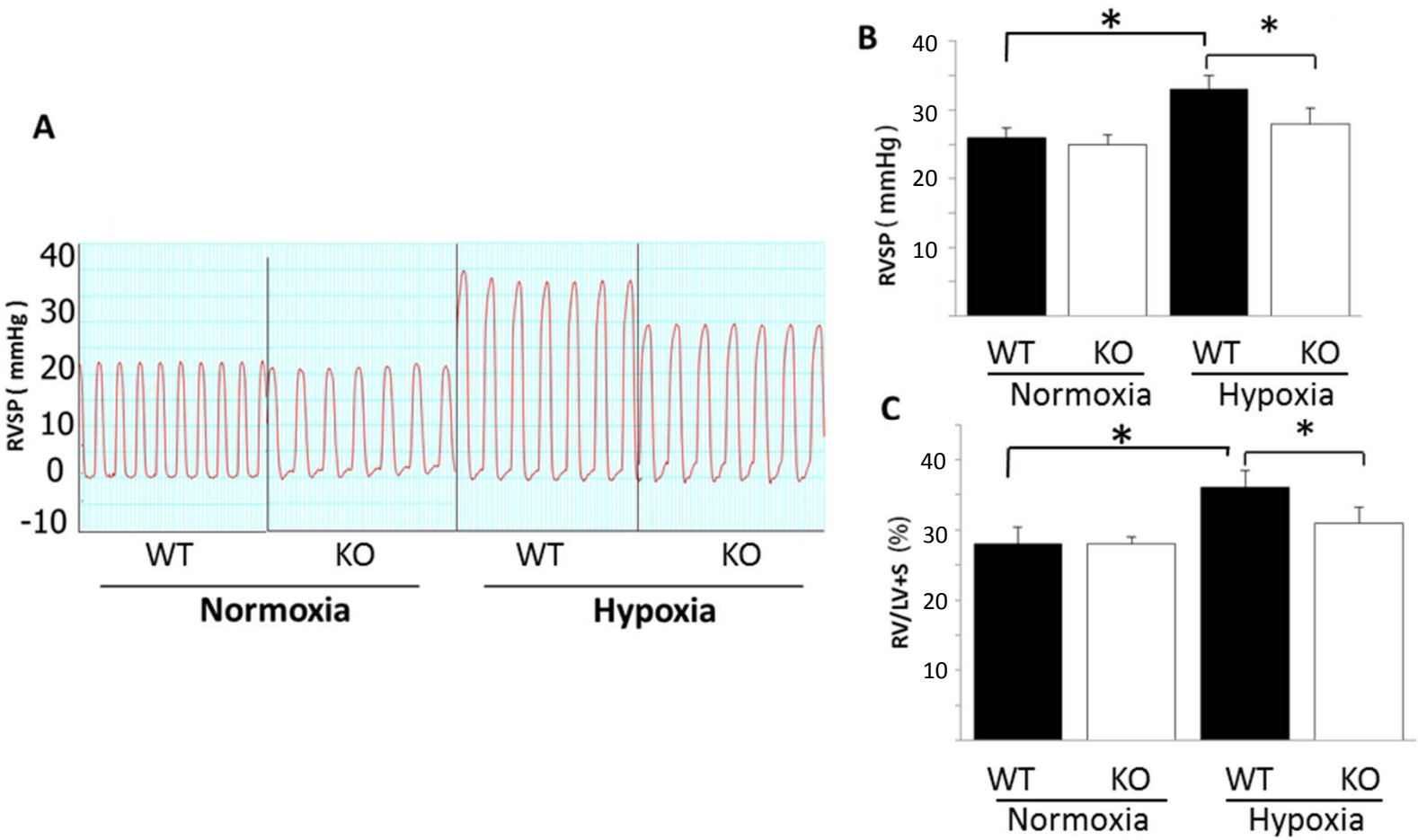
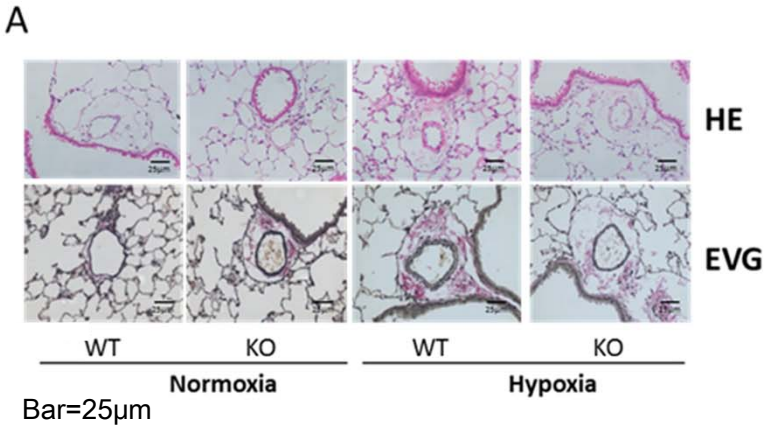
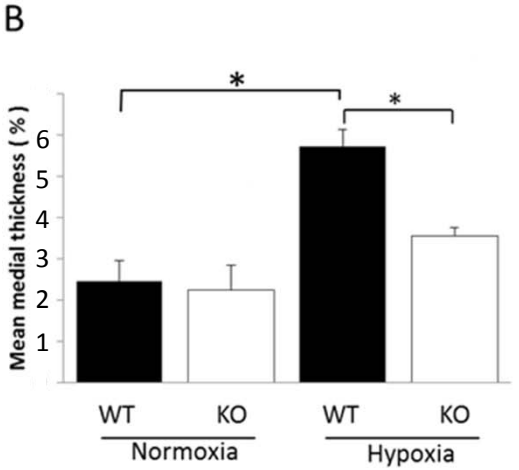
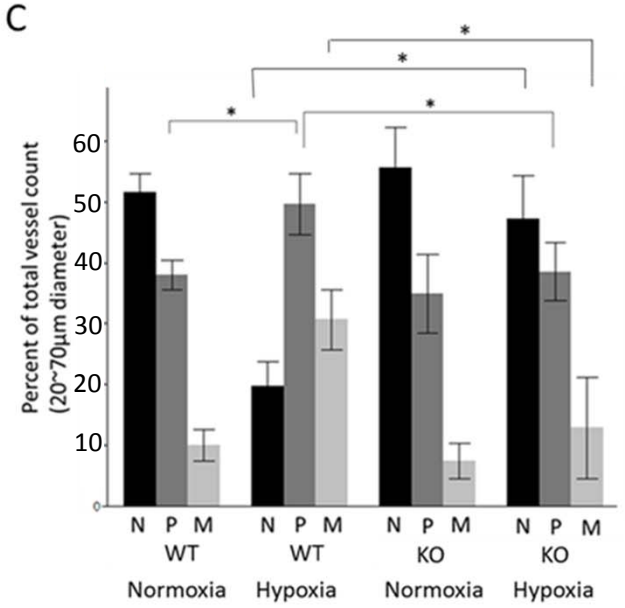

A
RVSP (mmHg)
40
30
20
10
0
-10
WT
KO
WT
KO
Normoxia
Hypoxia
B
RVSP (mmHg)
40
30
20
10
*
*
WT
KO
WT
KO
Normoxia
Hypoxia
C
RV/LV+S (%)
40
30
20
10
*
*
WT
KO
WT
KO
Normoxia
Hypoxia

Figure 3


A
HE
EVG
WT
KO
WT
KO
Normoxia
Hypoxia
Bar=25μm


B
Mean medial thickness (%)
1
2
3
4
5
6
*
*
WT
KO
WT
KO
Normoxia
Hypoxia


C
Percent of total vessel count
(20~70μm diameter)
0
10
20
30
40
50
60
*
*
*
*
N P M
WT
N P M
WT
N P M
KO
N P M
KO
Normoxia
Hypoxia
Normoxia
Hypoxia

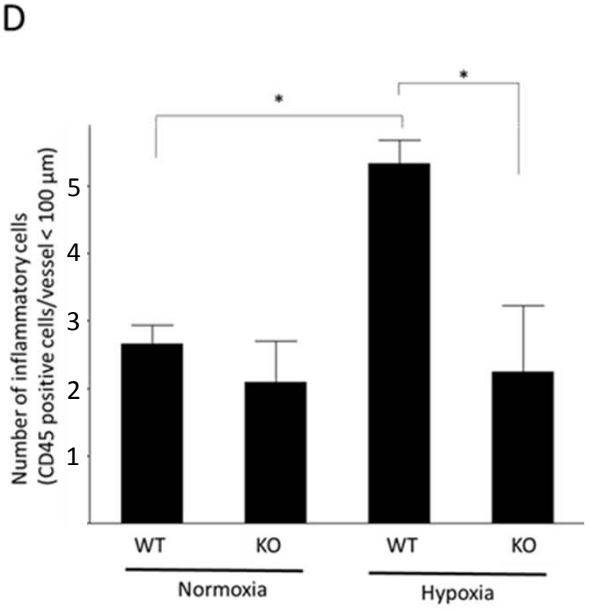

D
Number of inflammatory cells
(CD45 positive cells/vessel < 100 μm)
1
2
3
4
5
*
*
WT
KO
WT
KO
Normoxia
Hypoxia

Figure 4

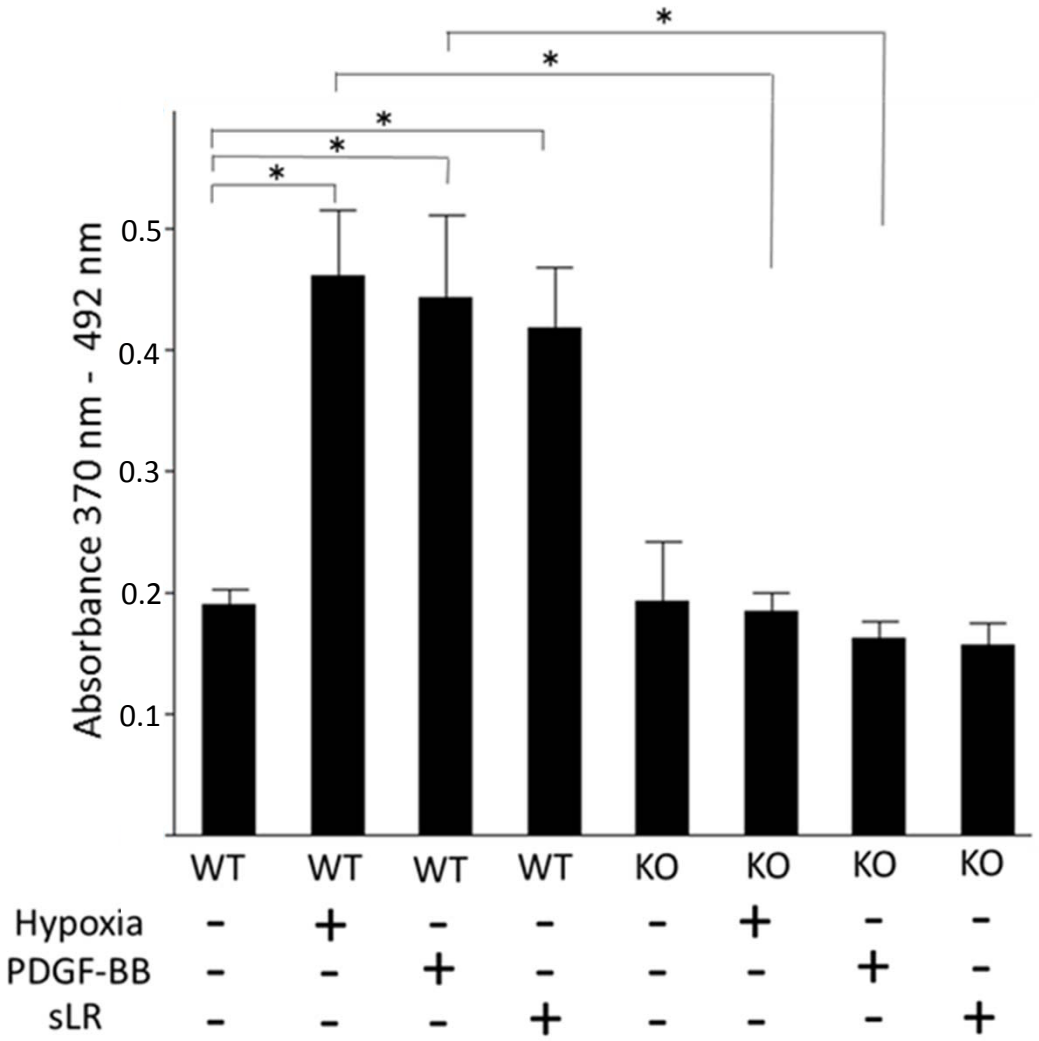

Absorbance 370 nm - 492 nm
0.5
0.4
0.3
0.2
0.1
*
*
*
*
*
WT WT WT WT KO KO KO KO
Hypoxia - + - - - + - -
PDGF-BB - - + - - - + -
sLR - - - + - - - +

Figure 5

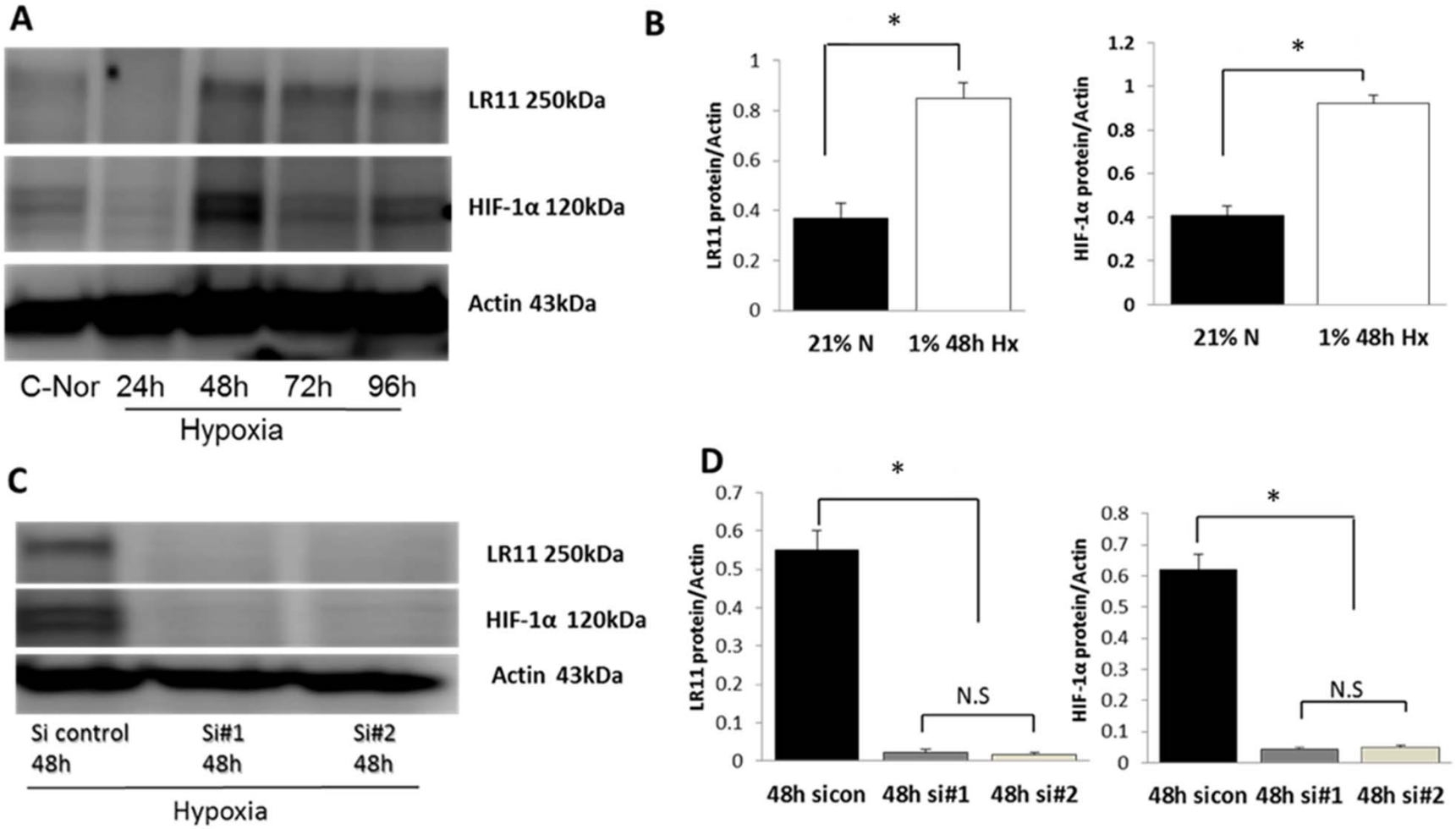
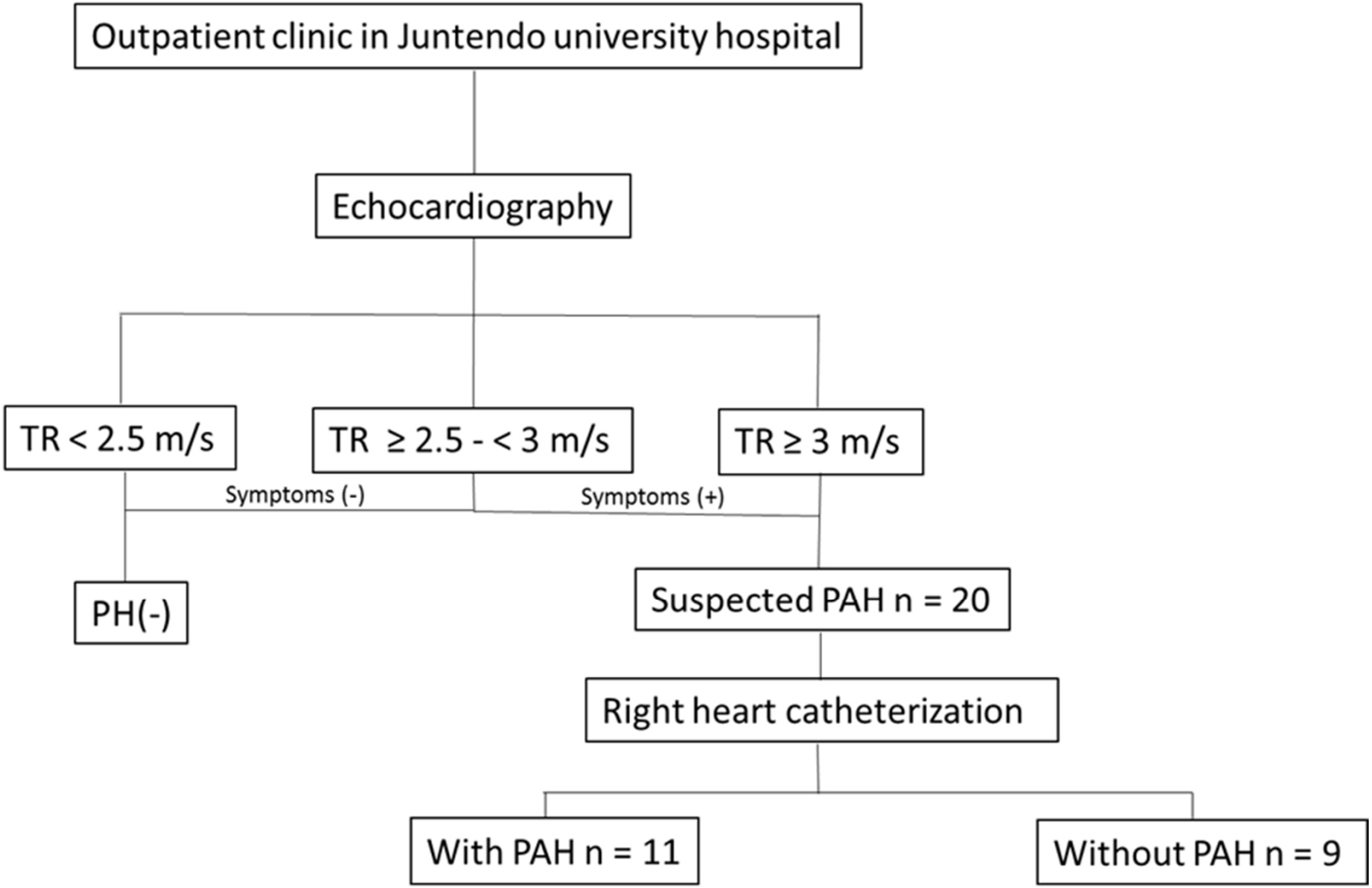

A
LR11 250kDa
HIF-1α 120kDa
Actin 43kDa
C-Nor 24h 48h 72h 96h
Hypoxia
B
LR11 protein/Actin
21% N
1% 48h Hx
HIF-1α protein/Actin
21% N
1% 48h Hx
C
LR11 250kDa
HIF-1α 120kDa
Actin 43kDa
Si control 48h
Si#1 48h
Si#2 48h
Hypoxia
D
LR11 protein/Actin
48h sicon
48h si#1
48h si#2
N.S
HIF-1α protein/Actin
48h sicon
48h si#1
48h si#2
N.S

Figure 6


Outpatient clinic in Juntendo university hospital
Echocardiography
TR < 2.5 m/s
TR ≥ 2.5 - < 3 m/s
TR ≥ 3 m/s
Symptoms (-)
Symptoms (+)
PH(-)
Suspected PAH n = 20
Right heart catheterization
With PAH n = 11
Without PAH n = 9