

Supplementary Information

Isolation and Characterisation of Lymphatic Endothelial Cells from Lung Tissues Affected by Lymphangioleiomyomatosis

Koichi Nishino^{1, 2*}, Yasuhiro Yoshimatsu^{3, 4}, Tomoki Muramatsu⁵, Yasuhito Sekimoto^{1, 2}, Keiko Mitani^{1, 2}, Etsuko Kobayashi^{1, 2}, Shouichi Okamoto^{1, 2}, Hiroki Ebana^{1, 2, 6, 7}, Yoshinori Okada⁸, Masatoshi Kurihara^{2, 6}, Kenji Suzuki⁹, Johji Inazawa⁵, Kazuhisa Takahashi¹, Tetsuro Watabe³, and Kuniaki Seyama^{1, 2}

¹ Division of Respiratory Medicine, Juntendo University Faculty of Medicine and Graduate School of Medicine

² Study Group for Pneumothorax and Cystic Lung Diseases

³ Department of Biochemistry, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University

⁴ Division of Pharmacology, Graduate School of Medical and Dental Sciences, Niigata University

⁵ Department of Molecular Cytogenetics, Medical Research Institute, Tokyo Medical and Dental University

⁶ Pneumothorax Research Center and Division of Thoracic Surgery, Nissan Tamagawa Hospital

⁷ Department of Thoracic Surgery, Tokyo Metropolitan Bokutoh Hospital

⁸ Department of Thoracic Surgery, Institute of Development, Aging and Cancer, Tohoku University

⁹ Department of General Thoracic Surgery, Juntendo University School of Medicine

***Corresponding author:**

Koichi Nishino

Division of Respiratory Medicine, Juntendo University Faculty of Medicine and

Graduate School of Medicine, 3-1-3 Hongo; Bunkyo-ku, Tokyo 113-8431, Japan

Tel: +81-3-5802-1063, Fax: +81-3-5802-1617, E-mail: k-nishino@juntendo.ac.jp

Supplementary Materials and Methods

Preparation of single-cell suspensions from lung tissues

Human lung tissues were processed to prepare single-cell suspensions as previously described¹. Briefly, lung tissues were cut into 2 to 3 g of specimen and put into 50-ml conical tubes containing neutral protease (2.0 U/mL Dispase II, Roche Applied Science, Penzberg, Germany), Collagenase/Dispase (1 mg/mL, Roche Applied Science), and deoxyribonuclease (0.1 mg/mL DNase I, Sigma-Aldrich, St. Louis, USA) in 50-ml conical tubes. The tissues were roughly minced by scissors in the conical tubes and incubated for 30 min with shaking (1 Hz). Next, the tissues were minced with scissors again and reincubated for another 60 min with shaking. Thereafter, the tissues were passed through an 18-gauge needle 5 times.

Subsequently, we added 10 ml of Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Waltham, USA) containing 10% fetal bovine serum and 100 U/ml of penicillin/streptomycin, and filtered the mixture through a Falcon 100- μ m Cell Strainer (Corning, Corning, USA) 2 times. After centrifugation at 1,500 revolutions/min (rpm) for 5 min, we discarded the supernatant and added 5 ml of Red Blood Cell Lysis Buffer (Roche Applied Science) to the cell pellet and continued incubation for 3 min at room temperature. After incubation, we added 15 ml of culture medium and centrifuged at

1,500 rpm for 5 min. We repeated the lysis step, followed by resuspension in 10 ml of culture medium and filtering 2 times through a Falcon 40- μ m Cell Strainer (Corning).

Supplementary Table S1. List of primers for qRT-PCR

Target Gene		Primer Sequence
<i>GAPDH</i>	Forward	TGATGACATCAAGAAGGTGGTGAAG
	Reverse	TCCTTGGAGGCCATGTGGGCCAT
<i>VEGFR-2</i>	Forward	CCTCCCCCGCATCACAT
	Reverse	GCTCGTTGGCGCACTCTT
<i>VEGFR-3</i>	Forward	TCTGCTACAGCTTCCAGGTGG
	Reverse	GCAGCCAGGTCTCTGTGGAT
<i>ITGA9</i>	Forward	CAAAGGCATCGGCAAGGTTT
	Reverse	TCCCCATTCAGGTCAACTGC

Supplementary Table S2. List of antibodies for immunohistochemistry

Antibody	Clone	Company	Dilution
Anti-human Calretinin	DAK-Calret-1	Dako A/S	1:50
Anti-human CK5/6	D5/16B4	Dako A/S	1:100
Anti-human podoplanin	D2-40	Dako A/S	1:200
Anti-human Prox-1	Rabbit Poly	AngioBio	1:1000
Anti-human LYVE-1	Rabbit Poly	Abcam	1:100
Anti-human VEGFR-3	Goat Poly	R&D Systems	1:50
Anti-human integrin $\alpha 9\beta 1$	2Q954	Santa Cruz Biotechnology	1:100

Supplementary Table S3. Up-regulated genes in LAM-LECs (T) regarding focal adhesion and the PI3K-AKT signaling pathway

Probe Name	Fold change	Gene symbol	Description
A_33_P3304668	7.510	<i>COL1A1</i>	Collagen, Type I, Alpha 1
A_24_P334300	6.351	<i>FGF12</i>	Fibroblast Growth Factor 12
A_23_P217319	5.947	<i>FGF13</i>	Fibroblast Growth Factor 13
A_24_P759477	3.895	<i>ITGB8</i>	Integrin, Beta 8
A_33_P3335725	3.874	<i>INSR</i>	Insulin Receptor
A_33_P3307267	3.425	<i>VWF</i>	Von Willebrand Factor
A_23_P65240	3.201	<i>COL4A1</i>	Collagen, Type IV, Alpha 1
A_23_P201636	2.603	<i>LAMC2</i>	Laminin, Gamma 2
A_23_P205031	2.521	<i>COL4A2</i>	Collagen, Type IV, Alpha 2
A_23_P60079	2.501	<i>ANGPT2</i>	Angiopoietin 2
A_32_P208403	2.426	<i>GNG2</i>	Guanine Nucleotide Binding Protein, Gamma 2
A_24_P124349	2.370	<i>PDGFD</i>	Platelet Derived Growth Factor D
A_23_P252193	2.363	<i>ITGA9</i>	Integrin, Alpha 9
A_24_P277934	2.355	<i>COL1A2</i>	Collagen, Type I, Alpha
A_24_P59667	2.306	<i>JAK3</i>	Janus Kinase 3
A_23_P153897	2.290	<i>GNG7</i>	Guanine Nucleotide Binding Protein, Gamma 7
A_23_P113005	2.248	<i>EFNA1</i>	Ephrina1
A_33_P3323959	2.244	<i>RELN</i>	Reelin
A_24_P339944	2.135	<i>PDGFB</i>	Platelet Derived Growth Factor Beta Polypeptide
A_33_P3212274	2.108	<i>F2R</i>	Coagulation Factor II Receptor
A_23_P156708	2.090	<i>TNXB</i>	Tenascin XB
A_23_P160968	2.048	<i>LAMC2</i>	Laminin, Gamma 2

Among the genes regarding cell adhesion and the PI3K-AKT -mTOR pathway, the up-regulated genes in LAM-LECs (T) with a fold change greater than 2 are listed in descending order, according to fold change calculated by GeneSpring software.

Supplementary Table S4. Up-regulated genes in control LECs regarding cell adhesion and the PI3K-AKT pathway

Probe Name	Fold change	Gene symbol	Description
A_23_P217379	22.356	<i>COL4A6</i>	Collagen, Type IV, Alpha 6
A_23_P45365	5.735	<i>COL4A5</i>	Collagen, Type IV, Alpha 5
A_33_P3305790	3.740	<i>NOS3</i>	Nitric Oxide Synthase 3
A_23_P404494	3.511	<i>IL7R</i>	Interleukin 7 Receptor
A_33_P3377364	3.382	<i>ITGB4</i>	Integrin, Beta 4
A_23_P338534	3.273	<i>HIF3A</i>	Hypoxia Inducible Factor 3, Alpha Subunit
A_23_P83818	3.024	<i>COL5A1</i>	Collagen, Type V, Alpha 1
A_23_P109269	3.004	<i>LAMA5</i>	Laminin, Alpha 5
A_23_P142187	2.800	<i>HIF3A</i>	Hypoxia Inducible Factor 3, Alpha Subunit
A_21_P0000025	2.706	<i>NOS3</i>	Nitric Oxide Synthase 3
A_33_P3629678	2.689	<i>COL5A1</i>	Collagen, Type V, Alpha 1
A_33_P3351944	2.655	<i>EGFR</i>	Epidermal Growth Factor Receptor
A_33_P3338121	2.576	<i>LAMB3</i>	Laminin, Beta 3
A_23_P89780	2.486	<i>LAMA3</i>	Laminin, Alpha 3
A_23_P166633	2.084	<i>ITGB5</i>	Integrin, Beta 5

Among the genes regarding cell adhesion and the PI3K- AKT-mTOR pathway, the upregulated genes in control LECs with a fold change greater than 2 are listed in descending order, according to fold change calculated by GeneSpring software.

Supplementary Table S5. Leading-edge genes of LEC-specific genes enriched in LAM-LECs (T)

Probe	Description	Rank Metric Score
<i>DSP</i>	Desmoplakin	3.610
<i>ALDH1A1</i>	Aldehyde Dehydrogenase 1 Family, Member A1	2.802
<i>HLA-DPB1</i>	Major Histocompatibility Complex, Class II, DP Beta 1	2.645
<i>MAF</i>	V-Maf Avian Musculoaponeurotic Fibrosarcoma Oncogene Homolog	2.398
<i>DCLK1</i>	Doublecortin-like Kinase 1	2.265
<i>RBP1</i>	Retinol Binding Protein 1, Cellular	2.101
<i>PEG10</i>	Paternally Expressed 10	2.099
<i>SEPP1</i>	Selenoprotein P, Plasma, 1	2.096
<i>CLEC4M</i>	C-Type Lectin Domain Family 4, Member M	2.094
<i>FABP4</i>	Fatty Acid Binding Protein 4, Adipocyte	1.987
<i>UNC5B</i>	Unc-5 Homolog B (C. Elegans)	1.915
<i>RGS16</i>	Regulator of G-Protein Signaling 16	1.914
<i>SOCS2</i>	Suppressor of Cytokine Signaling 2	1.894
<i>CH25H</i>	Cholesterol 25-Hydroxylase	1.887
<i>SLC26A4</i>	Solute Carrier Family 26 (Anion Exchanger), Member 4	1.876
<i>LAMP3</i>	Lysosomal-Associated Membrane Protein 3	1.742
<i>TIMP3</i>	TIMP Metallopeptidase Inhibitor 3	1.664
<i>RAMP3</i>	Receptor (G Protein-Coupled) Activity Modifying Protein 3	1.610
<i>ARID5B</i>	AT Rich Interactive Domain 5B (MRF1-Like)	1.606
<i>CDKN1C</i>	Cyclin-Dependent Kinase Inhibitor 1C (P57, Kip2)	1.560
<i>PCSK6</i>	Proprotein Convertase Subtilisin/Kexin Type 6	1.552
<i>MAOA</i>	Monoamine Oxidase A	1.465
<i>HOXD10</i>	Homeobox D10	1.451
<i>GPRC5B</i>	G Protein-Coupled Receptor, Class C, Group 5, Member B	1.443
<i>IGFBP2</i>	Insulin-Like Growth Factor Binding Protein 2, 36kDa	1.430

<i>SPRY1</i>	Sprouty Homolog 1, Antagonist of FGF Signaling (Drosophila)	1.353
<i>ANGPT2</i>	Angiopoietin 2	1.322
<i>IL7</i>	Interleukin 7	1.256
<i>NDRG1</i>	N-Myc Downstream Regulated 1	1.240
<i>ITGA9</i>	Integrin, Alpha 9	1.240
<i>RAMP2</i>	Receptor (G Protein-Coupled) Activity Modifying Protein 2	1.209
<i>PDPN</i>	Podoplanin	1.164
<i>RELN</i>	Reelin	1.139
<i>LMO2</i>	LIM Domain Only 2 (Rhombotin-like 1)	1.049
<i>CSRP2</i>	Cysteine and Glycine-Rich Protein 2	1.042
<i>CALCRL</i>	Calcitonin Receptor-like	1.016
<i>PRKCZ</i>	Protein Kinase C, Zeta	1.008
<i>SMAGP</i>	Small Cell Adhesion Glycoprotein	1.007
<i>LIPA</i>	Lipase A, Lysosomal Acid, Cholesterol Esterase	0.945
<i>ISG20</i>	Interferon- Stimulated Exonuclease Gene 20kDa	0.894
<i>MRC1</i>	Mannose Receptor, C Type 1	0.874
<i>PDE8A</i>	Phosphodiesterase 8A	0.842
<i>OLFML2A</i>	Olfactomedin-Like 2A	0.823
<i>CEACAM1</i>	Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (Biliary Glycoprotein)	0.777
<i>TUBA4A</i>	Tubulin, Alpha 4a	0.776
<i>PAQR3</i>	Progesterone and Adipoq Receptor Family Member III	0.750
<i>NR2F1</i>	Nuclear Receptor Subfamily 2, Group F, Member 1	0.745
<i>MEF2C</i>	Myocyte Enhancer Factor 2C	0.692
<i>SLC39A14</i>	Solute Carrier Family 39 (Zinc Transporter), Member 14	0.681
<i>PPP1R2</i>	Protein Phosphatase 1, Regulatory (Inhibitor) Subunit 2	0.677
<i>RMND5A</i>	Required for Meiotic Nuclear Division 5 Homolog A (S. Cerevisiae)	0.676
<i>KIAA0101</i>	Kiaa0101	0.674
<i>ADD3</i>	Adducin 3 (Gamma)	0.673
<i>BTBD3</i>	BTB (POZ) Domain Containing 3	0.660
<i>TK1</i>	Thymidine Kinase 1, Soluble	0.657

<i>PPAP2A</i>	Phosphatidic Acid Phosphatase Type 2A	0.632
<i>GCH1</i>	GTP Cyclohydrolase 1	0.605
<i>CTDSPL</i>	CTD (Carboxy-Terminal Domain, RNA Polymerase II, Polypeptide A) Small Phosphatase-like	0.540
<i>MYBL2</i>	V-Myb Avian Myeloblastosis Viral Oncogene Homolog-like 2	0.515
<i>REEP1</i>	Receptor Accessory Protein 1	0.514
<i>AURKB</i>	Aurora Kinase B	0.505
<i>DUSP5</i>	Dual Specificity Phosphatase 5	0.498
<i>LYN</i>	LYN Proto-Oncogene, Src Family Tyrosine Kinase	0.495
<i>ACSL3</i>	Acyl-CoA Synthetase Long-Chain Family Member 3	0.484
<i>GINS1</i>	GINS Complex Subunit 1 (Psf1 Homolog)	0.471
<i>TBX1</i>	T-Box 1	0.465
<i>RBPM5</i>	RNA Binding Protein with Multiple Splicing	0.465
<i>HIST1H4C</i>	Histone Cluster 1, H4c	0.455
<i>LMNB1</i>	Lamin B1	0.408
<i>FLT4</i>	Fms-Related Tyrosine Kinase 4	0.404
<i>CKS1B</i>	CDC28 Protein Kinase Regulatory Subunit 1B	0.403
<i>CCNA2</i>	Cyclin A2	0.401
<i>CETP</i>	Cholesteryl Ester Transfer Protein, Plasma	0.399

LEC-specific genes enriched in LAM-LECs (T) compared to control LECs are listed in descending order, according to the rank metric score calculated by Gene Set Enrichment Analysis software.

References

- 1 Ando, K. *et al.* Isolation of individual cellular components from lung tissues of patients with lymphangioleiomyomatosis. *Am J Physiol Lung Cell Mol Physiol* **310**, L899-908, doi:10.1152/ajplung.00365.2015 (2016).