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Isolation of individual cellular components from lung tissues of patients with lymphangioleiomyomatosis

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Ando K, Fujino N, Mitani K, Ota C, Okada Y, Kondo T, Mizobuchi T, Kurihara M, Suzuki K, Hoshika Y, Ebana H, Kobayashi E, Takahashi K, Kubo H, Seyama K. Isolation of individual cellular components from lung tissues of patients with lymphangioleiomyomatosis. Am J Physiol Lung Cell Mol Physiol 310: L899–L908, 2016. First published March 25, 2016; doi:10.1152/ajplung.00365.2015.—Lymphangioleiomyomatosis (LAM) is a rare neoplastic disease characterized by the proliferation of abnormal smooth muscle-like cells (LAM cells) that leads to cystic destruction of the lungs, chylous effusions, and the formation of lymphangioleiomyomas (21). This disease is found primarily in women of childbearing age and can occur either as a sporadic event (sporadic LAM) or as a pulmonary manifestation of tuberous sclerosis complex (TSC) (TSC-associated LAM) (8, 20, 21). LAM cells appear to result from the dysregulated mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) signaling, which is a key regulatory pathway of protein synthesis, cell growth, and energy metabolism, caused by mutations of either TSC1 or TSC2 genes (2, 5, 8). Recent clinical studies have successfully demonstrated that sirolimus, an mTOR inhibitor, stabilizes lung function and improves the quality of life in patients with LAM (1, 25). However, sirolimus might exert cytostatic rather than cytotoxic effects on LAM cells (25, 33), and its cessation causes a recurrence of declining progressive pulmonary function (25). In addition, Badri et al. recently examined exonic sequences of TSC genes in microdissected LAM cells by using a next-generation sequencer and reported that only a small fraction of LAM cells carry TSC mutations (3). At this point, the elucidation of further pathophysiological mechanisms and exploitation of new therapeutic agents are crucial issues.

The predominant histological features of LAM are the abundance of lymphatic vessels and the proliferation of LAM cells. Our previous pathological studies suggest that lymphangiogenesis-mediated fragmentation of LAM lesions and shedding of LAM cell clusters (LCCs) in the lymphatic stream likely play a role in the metastatic progression of LAM (23). Accordingly, the known intimate cellular interactions between LAM cells and lymphatic endothelial cells (LEC) need investigation to...
clarify and treat LAM’s pathological effects. However, no method exists for isolating LAM cells and other cellular components from LAM lesions for dissection of their interactions in vitro.

To date, laser capture microdissection has been used for the analyses of LAM-lung lesions. From results in previous studies with the microdissected samples, genetic alterations in the TSC genes were reported (3, 30). However, laser capture microdissection involves technical limitations in isolating individual cells, because not only LAM cells but also LEC and other cells are ensclosed within LAM lesions. Meanwhile, we recently established a technique for isolating individual cellular components from human lung tissue based on fluorescence-activated cell sorting (FACS) (12). With this technique, we isolated an individual alveolar component with high viability, i.e., alveolar type II cells (ATII), LEC, microvascular endothelial cells (VEC), mesenchymal cells (MS), and a cellular fraction consisting of alveolar type I cells (ATI) and bronchiolar epithelial cells. These isolations were achieved by using a combination of antibodies against epithelial cell adhesion molecule (EpCAM), podoplanin, and vascular endothelial (VE)-cadherin. Because LAM-affected lungs contain multiple LAM lesions, the advantage of this method is its usefulness for isolation of individual cells in LAM lesions, a process that we describe here in detail.

MATERIALS AND METHODS

Patients and preparation of tissue samples. LAM-affected lung tissues were obtained from patients who underwent lung transplantation at the Department of Thoracic Surgery, Tohoku University Hospital (Aobaku, Sendai, Japan) or video-assisted thoracoscopic surgery (VATS) for the treatment of pneumothorax at the Pneumothorax Research Center and Division of Thoracic Surgery, Nissan Tamagawa Hospital (Setagaya, Tokyo, Japan). We also obtained normal lung tissues from distal sites of tumors from patients who underwent lung resection for primary lung cancer at the Department of Thoracic Surgery, Juntendo University (Bunkyo, Tokyo, Japan).

In the operating room, those tissues were immediately immersed in 1 × 1 × 1 cm^3 (~2.0 grams) culture dishes and cut lung specimens into 1 × 1 × 1 cm^3 (~2.0 grams) on the clean bench. After being washed with phosphate-buffered saline, the tissues were immersed in new Stem Survive preservation solution (Stem Survive; Cell Science & Technology Institute, Sendai, Japan). Thereafter, we removed pleura bluntly in 10-cm culture dishes and cut lung specimens into 1 × 1 × 1 cm^3 (~2.0 grams) on the clean bench. After being washed with phosphate-buffered saline, the tissues were immersed in new Stem Survive solution and preserved at 4°C until use. To prepare frozen-tissue blocks, tissue sections were placed in a plastic mold (Tissue-Tek Cryomold; SFJ, Chuo, Japan) filled with frozen tissue matrix (O.C.T Compound; SFJ) and stored at −80°C until use. This study was approved by the ethics committee at each institution, and all subjects gave written informed consent.

Preparation of single-cell suspensions from lung tissue. Human lung tissues were processed to prepare single-cell suspensions as previously described with some modifications (13). We prepared single-cell suspensions and performed FACS experiments within 7 days after tissue preservation to obtain a high viability of lung cells. All steps except for enzymatic digestion at 37°C and mincing were performed at 4°C to increase cell viability. We first injected 2 ml of neutral protease (2.0 U/ml Dispase II; Roche Applied Science, Penzberg, Germany) in each lung specimen of 2.0 grams using a syringe with a 27-gauge needle and then placed it in a 50-ml conical tube containing 8 ml of Dispase II, 1 ml of collagenase/Dispase (1 mg/ml; Roche Applied Science), and 1 ml of deoxyribonuclease (0.1 mg/ml DNase I; Sigma-Aldrich, St. Louis, MO). The tissues were roughly minced by scissors in the conical tube and incubated for 30 min with shaking (60 Hz). After incubation for 30 min, the tissues were roughly minced with scissors again and reincubated for another 60 min with shaking. Thereafter, the tissues were further minced well with scissors and passed through an 18-gauge needle five times. After confirmation that the minced tissues would pass smoothly through the needles, we added 10 ml of culture medium [Dulbecco’s modified Eagle medium (Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum and 100 U/ml of penicillin/streptomycin] and filtered the mixture through a Falcon 100-μm Cell Strainer (Corning, Amsterdam, Netherlands) two times.

After centrifugation at 1,500 revolutions/min (rpm) for 5 min, we discarded the supernatant. We added 5 ml of red blood cell lysis buffer (Roche Applied Science, Penzberg, Germany) to the cell pellet and continued incubation for 3 min at room temperature. After incubation, we added 15 ml of culture medium and centrifuged the end product at 1,500 rpm for 5 min. We repeated the lysis step, followed by resuspension in 10 ml of culture medium and filtering two times through a Falcon 40-μm Cell Strainer (Corning).

Isolation of CD45− lung cells from single cell suspension. In single cell suspensions from LAM-affected lung tissues, we identified inclinations of not only CD45-expressing hematopoietic cells but also plentiful hemosiderin (24). To eliminate both and to obtain CD45− lung cells, we used the system of magnetic activated cell sorting and CD45 MicroBeads (Miltenyl Biotec, San Diego, CA) in accordance with the manufacturer’s instructions.

Flow cytometry and sorting of lung component cells. We used phycoerythrin (PE)-conjugated anti-human EpCAM antibody (clone 1B7; Biolegend, San Diego, CA, USA), PE-conjugated anti-human vascular endothelial growth factor receptor (VEGFR)-3 antibody (clone 3D9P; Biolegend), Alexa Fluor 467-conjugated anti-human podoplanin antibody (clone NC-08; Biolegend), fluorescein isothiocyanate (FITC)-conjugated anti-human VE-cadherin antibody (clone 55-7H1; BD Pharmingen, San Jose, CA), FITC-conjugated anti-human CD44v6 antibody (clone VFF-7; Thermo Fisher Scientific), FITC-conjugated anti-human CD34 antibody (clone 561; Biolegend), and Brilliant Violet 421-conjugated anti-human CD90 antibody (clone 5E10; Biolegend). We also purchased isotype-matched control antibodies from Biolegend. To discriminate between live and dead cells, we used 7-amino actinomycin D (7AAD) (eBioscience, San Diego, CA). We sorted live and single-cell-gated subpopulations using a FACS Aria II Cell Sorter (BD Biosciences, San Diego, CA). FACS analyses were performed using the FlowJo software package (Tree Star, Ashland, OR).

DNA/RNA extraction and reverse transcription-polymerase chain reaction. Genomic DNA and total RNA were isolated from cells collected by FACS using an All Prep Micro Kit (Qiagen, Venlo, Netherlands). Total RNA was reverse transcribed by using the SuperScript VILO cDNA Synthesis Kit (Thermo Fisher Scientific) in accordance with the manufacturer’s instructions. RNA of skin melanoma was purchased from the BioChain Institute (Hayward, CA) as a control template.

All primer sets used in this study are shown in Table 1. PCR was carried out using AmpliTaq Gold (Applied Biosystems, Foster City, CA) in a Bio-Rad iCycler (Hercules, CA) according to the manufacturer’s instructions as follows: denaturation at 94°C for 3 min followed by 40 cycles of 30 s at 94°C for denaturation, 30 s at 55°C for primer annealing, and 1 min at 72°C for polymerase extension, with a final elongation step at 72°C for 5 min. PCR products were separated by electrophoresis on 2% agarose gel.

PCR analysis of TSC2 loss of heterozygosity. To determine TSC2 loss of heterozygosity (LOH), isolated genomic DNA was amplified at the following six microsatellite markers; D16S521, D16S525, Kg8, D16S291, D16S663, and D16S283 on chromosome 16p13.3 as previously described (19). The samples were electrophoresed using an Applied Biosystems 3130/3130xl Genetic Analyzer (Applied Biosystems). LOH analysis was performed using a Gene Mapper 4.0 (Applied Biosystems), and a reduction in signal intensity over 50% was
Table 1. Primers used in the present study

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F: 5’-TGATGACATCAAGAAGTGTGAC-3’ R: 5’-TGCTGGAGCGGATCGGTATC-3’</td>
<td>240</td>
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<tr>
<td>gp100</td>
<td>5’-GCTGGGCACTCGGTCCACAT-3’ R: 5’-CACCGTGGACAGAAGACACG-3’</td>
<td>334</td>
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<tr>
<td>ER</td>
<td>F: 5’-TGGGAAGTGAAGATTGGGATAT-3’ R: 5’-GGTGGCCACCTCGTTCTGCT-3’</td>
<td>129</td>
</tr>
<tr>
<td>PR</td>
<td>F: 5’-GCAATGAGAGACACGACACAGGTTT-3’ R: 5’-GCAATGAGAGACACGACACAGGTTT-3’</td>
<td>320</td>
</tr>
<tr>
<td>CD90</td>
<td>F: 5’-GCGGTCGGACCAACGAGAAGCG-3’ R: 5’-AGTGGATGCTGTAATCCTC-3’</td>
<td>128</td>
</tr>
<tr>
<td>α-SMA</td>
<td>R: 5’-TGGCTATTTTCTTCTGGTACTA-3’ R: 5’-GATCCCAACAGACAGATTGGGC-3’</td>
<td>437</td>
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Table 2. Antibodies list for immunohistochemistry

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Clone</th>
<th>Companies</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-human α-SMA</td>
<td>IA4</td>
<td>Dako Cytomation</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-human melanoma-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>associated antigen gp100</td>
<td>HMB45</td>
<td>Dako Cytomation</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-human D2-40</td>
<td>D2-40</td>
<td>Dako Cytomation</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-human CD34</td>
<td>QBE8d10</td>
<td>Leica Biosystems</td>
<td>1:200</td>
</tr>
<tr>
<td>Anti-human CD90</td>
<td>SE10</td>
<td>Abcam</td>
<td>1:25</td>
</tr>
<tr>
<td>Anti-human Prox-1</td>
<td>Rabbit Poly</td>
<td>Angiobio</td>
<td>1:75</td>
</tr>
</tbody>
</table>

 defined as LOH. To confirm reproducibility, all experiments were examined at least two times.

**Immunofluorescence staining.** For immunofluorescence staining, the cytospun cells were fixed with 8% paraformaldehyde, blocked, and permeabilized. We immunostained EpCAM/podoplanin/CD34- cells with the following antibodies: α-smooth muscle actin (α-SMA) (clone IA4; Dako Cytomation, Carpinteria, CA) with Zenon Alexa Fluor 594 Mouse IgG2a Labeling Kits (Thermo Fisher Scientific) and melanoma-associated antigen gp100 (clone ab787; Abcam, Cambridge, UK) with Zenon Alexa Fluor 488 Mouse IgG1 Labeling Kits (Thermo Fisher Scientific) in accordance with the manufacturer’s instructions. Meanwhile, we used antibodies of α-SMA (clone IA4; Dako Cytomation) and surfactant protein-C (clone FL197; Santa Cruz Biotechnology, Dallas, TX) for immunostaining of cultured cells and the ATII subset, respectively. We also immunostained preserved tissue blocks using the following antibodies: α-SMA (clone IA4; Dako Cytomation), gp100 (clone HM245; Dako Cytomation), and CD90-biotin conjugated (clone SE10; Abcam) as the primary antibody and goat anti-mouse immunoglobulin (IgG2a, Alexa488) (Thermo Fisher Scientific), goat anti-mouse immunoglobulin (IgG1, Alexa488) (Thermo Fisher Scientific), and Alexa 594-conjugated streptavidin (Thermo Fisher Scientific) for the detection of the primary antibody binding, respectively. 4′,6-Diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) was used to stain nuclei. Immunofluorescence images were taken with Axioplan 2 Imaging (Zeiss, Oberkochen, Germany).

**Immunohistochemistry.** To prepare lung tissue blocks, small pieces of tissue were cut from resected lungs, fixed in 10% buffered formalin, and embedded in paraffin after routine processing. We stained lung specimens with hematoxylin-eosin or primary antibodies as shown in Table 2. As for immunostaining against CD90, we used preserved tissue blocks with O.C.T Compound. The EnVision+ System (Dako Cytomation) was used for the immunostaining of α-SMA, HM245, D2-40, CD34, and CD90, and prox-1 to detect the binding of the first antibody according to the manufacturer’s instruct-

Table 3. Clinical characteristics of patients with LAM in this study

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age</th>
<th>Tissue</th>
<th>FEV1, %pred</th>
<th>DLCO, %pred</th>
<th>Total Cells/LEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>Trans</td>
<td>70.2</td>
<td>31.0</td>
<td>10^6/10^9/12</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>Trans</td>
<td>38.0</td>
<td>16.9</td>
<td>10^6/8/25</td>
</tr>
<tr>
<td>3</td>
<td>41</td>
<td>Trans</td>
<td>25.9</td>
<td>9.2</td>
<td>10^6/5/10</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
<td>Trans</td>
<td>16.6</td>
<td>32.8</td>
<td>10^6/8/9</td>
</tr>
<tr>
<td>5</td>
<td>36</td>
<td>VATS</td>
<td>16.6</td>
<td>32.8</td>
<td>10^6/8/9</td>
</tr>
<tr>
<td>6</td>
<td>28</td>
<td>VATS</td>
<td>16.6</td>
<td>32.8</td>
<td>10^6/8/9</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>VATS</td>
<td>16.6</td>
<td>32.8</td>
<td>10^6/8/9</td>
</tr>
<tr>
<td>8</td>
<td>48</td>
<td>Trans</td>
<td>38.8</td>
<td>38.6</td>
<td>10^6/8/9</td>
</tr>
<tr>
<td>9</td>
<td>29</td>
<td>VATS</td>
<td>38.8</td>
<td>38.6</td>
<td>10^6/8/9</td>
</tr>
</tbody>
</table>

DLCO, carbon monoxide diffusing capacity; EpCAM, epithelial cell adhesion molecule; FEV1, forced expiratory volume in 1 s; LEC, lymphatic endothelial cells; Trans, transplantation; VATS, video-assisted thoracoscopic surgery; %pred, % predicted value of FEV1 and DLCO at the registration of lung transplantation. We could detect the EpCAM+/podoplanin− population in cases 2, 5, 7, and 8 (designated as “LAM” in Fig. 1B). Age indicates the age at the time of the operation. Data for Total Cells/LEC are the total no. of living cells and sorted LEC from 1 g of lung tissue.
LAM cells are heterogeneous not only morphologically but also immunohistochemically. The stainability of LAM-specific markers, such as HMB45, estrogen receptor (ER), or progesterone receptor (PR) is different in each LAM cell type. Among these markers, HMB45 has been used as an effective diagnostic marker for LAM (3, 21). Therefore, in this study, we focused on isolating HMB45-positive LAM cells.

We isolated separate subsets of ATII, MS, VEC, and LEC from patients 1 to 3 but confirmed mRNA expression of gp100 only in the MS subset (Fig. 1E). However, as shown in Fig. 1F, EpCAM+/podoplaninlow cells also expressed gp100 as well as ER, PR, α-SMA, and CD90. To the contrary, no TSC2 LOH was detected in any of these subsets (cases 1-3).

CD44v6-positive cells in MS subset. As previously reported, 1) CD44v6 was expressed in LAM lung lesions and 2) LAM

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**Fig. 1.** Fractionation of individual cellular components from normal and lymphangioleiomyoamatosi (LAM)-affected lung tissues. A: representative fluorescence-activated cell sorting (FACS) dot plot showing the expression of epithelial cell adhesion molecule (EpCAM) and podoplanin in CD45+ live lung cells from normal lung tissue (left) and the expression of vascular endothelial (VE)-cadherin in the EpCAM-/podoplaninpopulation (right). B: flow cytometry demonstrated that vascular endothelial growth factor receptor (VEGFR-3), a marker of lymphatic endothelial cells (LEC), was expressed by EpCAM-/podoplanin cells but not EpCAM+/podoplanin/VE-cadherin cells. C: immunofluorescence staining demonstrated that EpCAM+/podoplanin cells expressed surfactant protein (SP)-C, a marker of alveolar type II cells (ATII) (scale bar 20 μm). D: representative dot plot showing the expression of EpCAM and podoplanin in live CD45+ lung cells from LAM-affected lung tissue. A larger number of LEC was isolated from LAM-affected lung than from the same amount of normal lung. In samples from 4 of 9 LAM patients, we detected the EpCAM+/podoplaninlow population (designated as “LAM”). E: reverse transcription-polymerase chain reaction (RT-PCR) analysis (cases 1, 2, and 3) showed that gp100 was expressed in the isolated mesenchymal cell (MS) subset (EpCAM+/podoplanin/VE-cadherin) but not in the subsets of ATII (EpCAM+/podoplanin), microvascular endothelial cells (VEC; EpCAM+/podoplanin/VE-cadherin), or LEC (EpCAM+/podoplanin). F: mRNA expression of LAM-specific markers (case 2) was confirmed by RT-PCR in the isolated subset of EpCAM+/podoplaninlow cells (designated as LAM in D). ATII, alveolar type I cells; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LEC, lymphatic endothelial cells (replaced by “LY” in FACS dot plot); MS, mesenchymal cells; NC, negative control (deionized water used as a cDNA template); PC, positive control (RNA from skin melanoma used in the reaction); PR, progesterone receptor; VEC, microvascular endothelial cells (replaced y “VE” in FACS dot plot); α-SMA, α-smooth muscle actin.
cells with TSC2 LOH were detectable in not only cultured cells grown from explanted lungs but also bronchoalveolar lavage fluid, urine, and chylous effusions when reacted with anti-CD44v6 antibody (4, 28). Subsequently, we first tried to isolate CD44v6-positive cells from a sorted EpCAM⁺/podoplanin⁺/VE-cadherin⁻ (MS) subset. However, as shown in Fig. 2A, 7AAD, a marker for dead cells, was permeable by all CD44v6-positive cells in the MS subset. This result was similar to that in a previous study (28). Although the total number of 7AAD-positive cells when combined with anti-CD44v6 antibody did not increase compared with that in combination with its iso-type-matched control antibody, we concluded that this method was not applicable for the isolation of live LAM cells.

HMB45-positive LAM cells are CD34 negative and CD90 positive. The MS subset we previously isolated consisted mainly of fibroblasts in alveolar walls, and, when cultured, these MS cells expressed mesenchymal markers such as α-SMA and CD90 (12). Others have reported that lung fibroblasts expressed not only CD90 but also CD34 (16, 29). Therefore, we next assessed CD34 and CD90 expression in MS (EpCAM⁺/podoplanin⁺/VE-cadherin⁺) subsets.

As shown in Fig. 2B, our immunohistochemical examination revealed the immunopositivity for CD34 in fibroblasts from alveolar walls and D2-40-positive LEC in LAM lesions, whereas α-SMA-positive LAM cells were negative for CD34. Similarly, we found that VEC subsets sorted from normal and also LAM lungs were positive for CD34 (Fig. 2C). Most cells (90.0 ± 0.8%) in the MS subset from normal lungs were positive for CD34 (n = 2); however, the proportion of CD34⁺ cells in the MS subset from LAM lungs was low (29.6 ± 1.7%, n = 2) (Fig. 2D). Finally, no gp100 mRNA was detected in CD34⁺ cells of the MS subset from LAM lungs (Fig. 2E),

Fig. 2. Flow cytometric analyses and expression of CD34 and CD90 in LAM lung and MS subset. A: 4 populations of cells were defined in the LAM lung-derived MS subset by reaction with 7-amino actinomycin D (7AAD) and CD44v6-FITC antibody. Compared with the result of isotype-matched control analysis (left), the total number of 7AAD-positive cells did not increase when immunostained by anti-CD44v6 antibody (right), indicating that 7AAD was permeable by CD44v6⁺ cells. B: immunohistochemistry of LAM lung tissue showing expression of podoplanin (D2-40), α-SMA, and CD34. HE denotes hematoxylin-eosin staining. Note that α-SMA-positive LAM cells were negative for CD34. C: flow cytometric analysis revealed that the VEC subset isolated from LAM-affected lung tissue expressed CD34; the VEC subset isolated from normal lung showed the same result (flow cytogram not shown). D: flow cytometric analysis of MS subset (EpCAM⁺/podoplanin⁺/VE-cadherin⁻) cells. The proportion of CD34⁺ cells increased in the MS subset from LAM-affected lung compared with that from normal lung. E: expression of gp100 in the CD34⁺ MS population (EpCAM⁺/podoplanin⁺/VE-cadherin⁺/CD34⁺) was not demonstrated by RT-PCR. Top, immunofluorescence staining for α-SMA (green), CD90 (red), DAPI (blue), and merged view (from left to right). Note colocalization of α-SMA and CD90 expression. Similarly, the bottom shows the staining for HMB45 (green), CD90 (red), DAPI (blue), and the merged view (from left to right). Most of the HMB45-positive cells expressed CD90.
suggesting that LAM cells must be included in the CD34− MS population.

Next, immunohistochemistry and immunofluorescence staining confirmed that the majority of α-SMA+ and HMB45+ LAM cells were also positive for CD90 (Fig. 2F). Flow cytometric analysis supported that CD34+ cells in an MS subset from LAM lungs included CD90+ cells (Fig. 3A). In the CD34− MS subset from normal lungs, very few cells were positive for CD90, i.e., only 2.7 ± 1.5% of cells were CD90+ (n = 3) (Fig. 3A, left). This result is consistent with previous pathological studies (16, 29). In contrast, CD34+ cells in the MS subset from LAM lungs contained CD90+ cells (11.0 ± 5.1%, n = 5) (Fig. 3A, middle). Similarly, CD90+ cells were found in EpCAM−/podoplaninlow populations from LAM lungs (Fig. 3A, right).

EpCAM−/podoplaninlow/CD34−/CD90+ cell populations include HMB45-positive and TSC2 LOH-harboring LAM cells. We next examined whether EpCAM−/podoplaninlow/CD34−/CD90+ cells from LAM-affected lungs (1) express gp100 and 2) harbor TSC2 LOH. Here, we mainly used lung tissues from patients who underwent VATS for pneumothorax (cases 5-7). The EpCAM−/podoplaninlow subset was obtained only from case 7.

As Fig. 3B illustrates, CD90+ cells from the EpCAM−/podoplaninlow/CD34− subset highly expressed gp100. Similarly, gp100 was expressed in CD90+ cells from the EpCAM−/podoplaninlow subset (case 7). In contrast, CD90− cells from both EpCAM−/podoplanin−/CD34− and EpCAM−/podoplaninlow subsets faintly expressed or did not express gp100 at all.

We next searched for α-SMA and HMB45 in EpCAM−/podoplaninlow/CD34−/CD90+ cells by applying immunofluorescence stain (Fig. 3C). α-SMA was expressed by 85.0 ± 7.7% of EpCAM−/podoplaninlow/CD34−/CD90+ cells and HMB45 by 80.0 ± 2.0% of cells (n = 3, cases 7-9). On the other hand, expression of these LAM-related markers was low in CD90− cells; 20.1 ± 9.8% of cells were positive for α-SMA, whereas 6.6 ± 9.4% of these cells expressed α-SMA.

![Figure 3](http://ajplung.physiology.org/)
HMB45. Finally, TSC2 LOH was demonstrated in EpCAM+/podoplanin-/CD34+/CD90+ cells (cases 5-7) (Figs. 3D, 3E, and 4D).

Characteristics of EpCAM+/podoplanin−/low/CD34+/CD90+ cells and the LEC subset in culture. We examined in vitro the behaviors of EpCAM+/podoplanin−/low/CD34+/CD90+ cells and the LEC subset. Cells derived from the EpCAM+/podoplanin-/CD34+/CD90+ population in case 6 attached onto culture slides and displayed a spindle shape on day 10 (Fig. 4A). Reverse transcription-polymerase chain reaction (RT-PCR) analysis demonstrated that they expressed gp100, ER, and PR on day 10 but not on day 20 (2 more passages after day 10) (Fig. 4B). However, these cells continued to express both α-SMA and CD90 on day 20 (Fig. 4C). By day 20, TSC2 LOH was no longer detectable in these cultures (Fig. 4D). We next examined the ability of cultured cells on day 20 to proliferate and invade compared with that of the normal MS subset. As shown in Fig. 4, E and F, those abilities were similar in both types of cells. However, it remains unclear whether these findings faithfully reflect the abilities of EpCAM+/podoplanin−/low/CD34+/CD90+ just after the isolation by FACS. Regarding the LEC subset (EpCAM+/podoplaninhi cells), these cells were reproducibly amenable to culture and maintained their LEC features in terms of cell appearance and expression of podoplanin (D2-40) and prox-1 (Fig. 4G).

DISCUSSION

The study detailed here is a successful application of our unique FACS-based method (12) for isolating separate cellular components from LAM tissues obtained from lungs of transplant and VATS recipients. Our method has several notable advantages over those previously used to isolate LAM cells. For example, formerly, laser-captured or manual dissection of

Fig. 4. Culture of EpCAM+/podoplanin+/VE-cadherin+/CD34−/CD90+ LAM cells and LEC subset. A: EpCAM+/podoplanin+/VE-cadherin+/CD34−/CD90+ LAM cells displayed a spindle shape when cultured in smooth muscle growth medium (day 10; scale bar 100 μm). B: RT-PCR analysis demonstrated that mRNA expression of gp100, ER, and PR by EpCAM+/podoplanin+/VE-cadherin+/CD34−/CD90+ LAM cells was maintained on day 10 but disappeared on day 20 after the initiation of culture. C: despite the disappearance of LAM characteristics in CD90+ LAM cells, immunofluorescence staining and flow cytometric analysis revealed that expression of α-SMA and CD90 was maintained, respectively (day 20; scale bar 100 μm). D: sorted EpCAM+/podoplanin+/VE-cadherin+/CD34−/CD90+ cells harbored TSC2 LOH at the D16S525 locus (case 6), but it disappeared on day 20 after cultivation. E: proliferation of cultured EpCAM+/podoplanin+/VE-cadherin+/CD34−/CD90+ LAM cells (case 6). LAM cells were extended in 4 passages after FACS sorting and then used for proliferation assay that was performed in triplicate. Cultured MS subset obtained from normal lung was used as a control. (n = 3). Data are expressed as means ± SD. F: invasion assay of cultured EpCAM+/podoplanin+/VE-cadherin+/CD34−/CD90+ LAM cells (case 6). The conditions of LAM cells and control normal MS subset (n = 3) were the same as those in E. Data are expressed as means ± SD. G: cultured cells from the EpCAM+/podoplaninhi population displayed a plump cuboidal shape (left). Immunocytochemistry showed that they were immunopositive for D2-40 (middle) and prox-1 (right) (day 30; scale bar 200 μm).
LAM cells from frozen or formalin-fixed pathological specimens was used for genetic or molecular analyses, but those processes are time consuming and labor intensive. In addition, live LAM cells could not be obtained. Although live LAM cells can be derived from cultures of excised LAM lung tissues (11) or isolated from circulating blood, other body fluids such as chylous effusion, bronchioalveolar lavage fluid, and urine (4, 28), no other cellular components of LAM lesions can be isolated simultaneously. Alternatively, LCC, globular structures consisting of LAM cells and LAM-associated LEC, are easily isolated from chylous effusions of LAM patients (23, 26). LCC, although alive and able to grow and expand in culture, are difficult to separate LAM cells and LEC individually. As an improvement, the FACS-based method we describe in this study enabled us to collect live cellular components from LAM lesions (Fig. 5), thereby providing an opportunity to investigate cell-cell interactions within such lesions.

In the present study, we defined LAM cells as cells positive for HMB45 and harboring TSC2 LOH. The expression of melanosome-related proteins and genetic alterations have been recognized as pathognomonic characteristics of LAM (3, 21, 23). However, LAM cells are likely to be heterogeneous populations in terms of cell morphology and genetics. Microscopically, LAM cells are smooth muscle-like cells with either an epithelioid or spindle-shaped morphology (18). Immunopositivities for α-SMA, HMB45, ER, and PR are important pathological identifiers in the diagnosis of LAM, but not all LAM cells express these immunohistochemical markers (21, 23). Furthermore, Badri et al. recently reported that not all LAM cells have the TSC2 mutation; allelic frequencies of the TSC2 mutation ranged from 4 to 60% with most at <20% (3). In addition, the LAM cells of two patients included in their study were immunopositive for both hamartin and tuberin as well as immunonegative for phospho-S6 kinase, indicating no evidence of mTORC1 activation. Accordingly, in the current study, our combined use of antibodies against EpCAM, podoplanin, CD34, and CD90 resulted in the isolation of HMB45-positive LAM cells with TSC2 LOH, but we need to realize that other types of LAM cells, for example, HMB45-negative LAM cells, exist in other cellular fractions, especially in the MS subset.

Although the cellular origin and normal counterparts of LAM cells remain unknown, Delaney et al. hypothesized that LAM cells originate from the neural crest, since molecular markers for mesenchymal neural crest cells such as HMB45 and melanoma antigens recognized by T cells are expressed by LAM cells (10). CD90 is one of the mesenchymal stem cell markers, also called thymocyte differentiation antigen 1. We previously reported that CD90 is expressed by lung fibroblasts in histopathological specimens and cells cultured from the MS subset (12, 13). Furthermore, LAM cells grown from chylous effusions also expressed CD90 (15). Additionally, lung fibroblasts derived from CD34+ mesenchymal stem cells proved to be positive for CD34 (16, 29). When examining normal lungs, we found that the majority of cells in the MS subset were CD34+; conversely, in the MS subset from LAM-affected lungs, HMB45-expressing cells were CD34+. These findings suggest that some surface markers in LAM cells overlap other lung mesenchymal cells. Interestingly, Clements et al. reported that LAM cells express fibroblast surface protein and fibroblast activation protein, thus indicating the possibility that wild-type fibroblast-like cells might form LAM-lung nodules (7). Conceivably, then, LAM nodules contain mixtures of mesenchymal cells that share the features of fibroblasts but originate from other sites or differentiate in several directions.

We identified two LAM cell populations in terms of podoplanin expression: EpCAM+/podoplanin+ and EpCAM+/podoplanin−. The former were isolated from all of the LAM patients we examined, but the latter appeared in only four of nine patients (cases 2, 5, 7, and 8). The immunostaining intensity of podoplanin was at the same level as that of ATI. Podoplanin is a mucin-like transmembrane glycoprotein reportedly expressed by not only LEC and ATI but also by a variety of malignant tumors (31). Previously, Hansen et al.

![Fig. 5. Schema of the method for isolating multiple cell populations from LAM-affected lung tissues.](http://ajplung.physiology.org/)
reported that LAM cells were immunonegative for D2-40, a monoclonal antibody directed against podoplanin (17). In contrast, others found that the majority of LAM cells were immunopositive for D2-40, but its intensity varied and was weaker than that by LEC (9, 14). Therefore, the biological significance of podoplanin expression in LAM cells is unclear and remains controversial. When Suzuki et al. used podoplanin-overexpressing lung cancer cell lines to investigate its role in tumor progression, podoplanin downregulated the expression of VEGF-C, and VEGF-D then suppressed the tumor’s lymphangiogenesis and lymph node metastasis (34). In this context, the possibility exists that podoplanin− LAM cells have a greater potential for metastasis than podoplaninlow LAM cells.

Fuller investigation of the role of cell-cell interaction is undoubtedly required for understanding LAM pathology, as illustrated by Clements et al. (7). They implicated fibroblasts as an important group of cells that would associate with LAM cells. We believe that another important cellular component with which LAM cells interact and form LAM lesions is LEC, since LAM cells produce VEGF-D, an inducer of LAM-associated lymphangiogenesis (26, 32). Zeng et al. noted that the interaction of tumor cells with LEC modulates LEC behavior and is related to the ability of tumor cells to form lymph node metastases (35). This finding could be extrapolated to account for the mechanisms of disease progression in LAM. In fact, we recently reported that LCCs become a useful model for analyzing the interaction between LAM cells and LEC when cultured in a three-dimensional collagen gel system (22). Because our FACS-based method, for the first time, enables us to isolate LAM cells and LEC separately, their interaction can now be investigated through the reconstitution of LCCs in vitro. Before accomplishing this end, however, we must first solve the following problem. We found that the expression of gp100, ER, and PR rapidly disappeared once EpCAM−/podoplanin−low/CD34+ /CD90+ cells were placed in the culture system, although they maintained the expression of CD90 and α-SMA. In addition, TSC2 LOH1 was no longer present by 20 days after the initiation of culture. Considering that EpCAM−/podoplanin−low/CD34+/CD90+ cells harbor the TSC2 mutation, they would be expected to show a robust growth advantage through dysregulated mTORC1 activation. However, our data suggest that cells without the TSC2 mutation, presumably contaminants, proliferated faster in vitro than expected. The discrepancy between the growth pattern we encountered in vitro and the expectation from in vivo molecular profiling might be caused from their differing environmental influences (6, 27). Alternatively, some of the EpCAM−/podoplanin−low/CD34+/CD90+ cells could have stem cell properties and require the niche microenvironment to maintain LAM cell characteristics. For example, in TSC-associated lesions such as facial angiofibromas, cells that originated from neural crest stem cells and their precursors have been found (10). In conclusion, we have established a novel FACS-based method for the direct isolation of individual LAM cell populations and related pulmonary cells. Isolating and culturing LAM cells and LAM-associated LEC from LAM-affected tissues by using this method enables us to perform much-needed, cell-specific analyses. Consequently, our FACS-based method constitutes a valuable tool for research destined to ameliorate the disease process of LAM.

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DISCLOSURES

The authors have reported that no potential conflicts of interest exist with any companies/organizations whose products or services may be discussed in the article.

AUTHOR CONTRIBUTIONS


REFERENCES


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26. Mitani K, Kumasaka T, Takemura H, Hayashi T, Gunji Y, Kunogi M, Akiyoshi T, Takahashi K, Suda K, Seyama K. Cytologic, immunocytochemical and ultrastructural characterization of lymphangiomyomatosis cell clusters in chylous effusions of patients with lymphangiomyo-


