Mitochondrial dysfunction is a key determinant of the rare disease lymphangioleiomyomatosis and provides a novel therapeutic target

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Abstract
Lymphangioleiomyomatosis (LAM) is a rare and progressive systemic disease affecting mainly young women of childbearing age. A deterioration in lung function is driven by neoplastic growth of atypical smooth muscle-like LAM cells in the pulmonary interstitial space that leads to cystic lung destruction and spontaneous pneumothoraces. Therapeutic options for preventing disease progression are limited and often end with lung transplantation temporarily delaying an inevitable decline. To identify new therapeutic strategies for this crippling orphan disease, we have performed array based and metabolic molecular analysis on patient-derived cell lines. Our results point to the conclusion that mitochondrial biogenesis and mitochondrial dysfunction in LAM cells provide a novel target for treatment.

Introduction
Ten-year survival of the progressive and systemic orphan disease, Lymphangioleiomyomatosis (LAM) (1–9/1,000,000 adult women) [1], ranges from 40% to 79% [2], but these figures do not fully capture the significant and debilitating reduction in life-quality experienced by most sufferers. While a number of organs can be affected, including the kidney and associated lymph nodes, it is the deterioration in lung function, which leads to disease progression. The early disease symptoms of LAM show high similarity to asthma, chronic obstructive pulmonary disease, and other obstructive lung diseases; therefore, the condition is frequently misdiagnosed [3]. Only through a combination of computer tomography, serological testing of increased serum vascular endothelial growth factor-D (VEGFD), and matrix metalloproteinase (MMP) levels, and lung biopsy, can clinicians confirm a LAM diagnosis with confidence [4]. Although the progression of the disease is relatively slow, most patients suffer from accelerating respiratory failure and can experience decades long dyspnea before lung transplantation is considered as a “last-resort” therapy. LAM is caused by inherited (TSC-LAM) or acquired (sporadic or S-LAM) mutations of the tumor suppressor tuberous sclerosis complex (TSC) genes TSC1 (hamartin) or TSC2 (tuberin) [3]. The TSC1–TSC2 complex interacts with various signaling pathways and is also involved in regulation of the mechanistic target of Rapamycin.
(mTOR1) complex (mTORC1) via stimulation of GTPase activity of the small GTPase Rheb [5]. Although the majority of LAM tissues characteristically carry TSC1 or TSC2 mutations, a significant number of cases (10–15%) still present with no mutations in the TSC genes, suggesting undetected mutagenic events or deregulation of signaling pathways via an alternative route [6] which possibilities are actively researched in leading laboratories of the field [7].

Currently, treatment options remain limited to the mTOR inhibitor Rapamycin (Sirolimus) [8] that stabilizes lung function in most patients but does not offer progression-free survival.

As LAM occurs almost exclusively in women, some clinical studies have claimed that control of serum estrogen levels offers up an alternative route to preventing disease progression [9]. Although such attempts have mostly failed [9], we theorized that it was not the original idea but the study approach that might have been unsuccessful. In an attempt to understand more fully the broader disease mechanisms (in addition to TSC mutations) that might mediate LAM pathogenesis, we have performed TaqMan and Nanostring array analysis of LAM cells isolated directly from the lungs of transplant patients [10]. Through a combination of techniques, we have identified gene expression profiles and miRNA signatures that strongly indicate that an alteration in mitochondrial biogenesis and function are among the key determinants behind increased VEGF production and accelerated cell proliferation. To test our conclusions, we have also treated LAM cell lines with the potent, mito-targeting and mito-active drug candidate Proxison [11] and observed restoration of mitochondrial function and a corresponding reduction in VEGF production and proliferation capacity.

Results

Deregulation of nuclear receptors, vascularization, and miRNAs in LAM

Estrogen hormone affects cellular signaling and metabolism via two receptor types located on the cell membrane (Estrogen Receptor A or ERA) and within the nucleus (Estrogen Receptor B or ERB) [12]. A nuclear receptor array was performed using pooled samples of two individual bronchial smooth muscle cell (SMC) controls and pooled samples from four LAM patient-derived cell lines (Fig. 1a) (Supplementary Table 1 and Fig. 1). Of the 92 examined genes, 21 have shown an increase, and a further 30 a reduction in expression levels when compared to normal SMCs. The nuclear receptor TaqMan array (Fig. 1b) and consequent quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis on individual cell types (Supplementary Fig. 2) identified the progesterone receptor (PGR), the peroxisome proliferator-activated receptor gamma coactivator 1-beta (PPARGC1B) as being significantly overexpressed, and estrogen-related receptor gamma (ESRRG) as markedly upregulated. PPARGC1B is known to regulate the transcriptional activity of the estrogen receptor alpha (ERA), nuclear respiratory factor 1 (NRF1), and glucocorticoid receptor (GR) genes. The expression of each of these genes increased over seven-fold in the LAM cell lines tested (Fig. 1b). PPARGC1B overexpression is linked to increased mitochondrial number [13, 14], the active PRG isof orm 4 to increased mitochondrial membrane potential and cellular respiration [13, 14], while ESRRG to control of mitochondrial biogenesis and energy metabolism [6, 14]. The significantly down-regulated nuclear receptor genes included NR5A2 and retinoic acid receptor beta (RARB). RARB is a member of thyroid-steroid hormone receptor superfamily (Fig. 1b) and both genes are known to play a powerful role in the inhibition of proliferation and stimulation of cellular differentiation [15]. Artificial Neural Network (ANN) analysis [16] of the data sets revealed a strong positive correlation with several retinoic acid receptors (RARB, RXRB, and RXRG) all of which bind the biologically active form of vitamin A and PGR (Fig. 1b). Changes in nuclear receptor gene expression in the disease cell lines directly point towards strong mitochondrial involvement in LAM linking data to previous studies demonstrating that LAM cell proliferation, driven by mTOR activation, requires major adjustments in energy metabolism [17]. Instead of utilizing NADP-driven oxidative phosphorylation, mitochondrial energy production by LAM sufferers (also seen in some cancers) is predominantly limited to aerobic glycolysis (Warburg effect) [17]. Such changes in energy metabolism lead to an increased expression of the hypoxia-inducible factor 1 alpha (HIF1-alpha) [17, 18] (Fig. 1c), and consequently to increase in VEGF expression [19]. In support, qRT-PCR analysis (Fig. 1c) and an angiogenesis protein array (Fig. 1c) detected strong up-regulation of LAM diagnostic markers VEGFC and VEGFD (Fig. 1c). A decrease in thrombospondin-1 (TSP1) and an increase in CXCL16 chemokine peptide levels were also observed in the LAM samples (Fig. 1d). These observations are particularly interesting as TSP1 is a recognized inhibitor of mitochondrial biogenesis [20], while CXCL16 regulates cellular invasion in non-small-cell lung cancer (NSCLC) [21]. ANN analysis of the angiogenesis array data (Fig. 1e) identified a strong association of CXCL16 and TSP1 with the fibroblast growth factor (FGF), Endothelin1, SerpinE1, and VEGFC (Fig. 1e) levels that are all
Mitochondrial dysfunction is a key determinant of the rare disease lymphangioleiomyomatosis and... involved in the stimulation of vascularization and the induction of myofibroblastic phenotype that in itself can reduce the capacity for pulmonary regeneration [22].

Further studies using a Nanostring methodology identified down-regulation of both the tumor suppressor miR125b-5p [23] and the low-density lipid oxidation induced autophagy regulator miR155-5p [24] in LAM. The apoptosis inducer miR-15b-5p [24] was down-regulated, while the cell proliferation and survival inducer miR-199a/b-3p [24] had increased copy numbers in individual LAM cell samples (Fig. 1f).

Based on the data above, compromised mitochondrial activity is appeared to be an additional factor in LAM disease progression.

**Mitochondrial dysfunction in LAM**

Electron microscopic images of the normal SMC and LAM cell lines showed a drastically different mitochondrial activity.
Mitochondria in LAM cells were smaller, darker, and so electro-dense that the inner membrane cristae were not visible (Fig. 1a). The gene profiling data again support these microscopic observations. NRF1 encodes a homodimerizing protein, which functions as a transcription factor for key metabolic genes required for cellular growth, respiration, mitochondrial DNA transcription, and replication. NRF1 was higher in LAM than in control SMCs (Fig. 2a) and has previously been linked to PPARGC1B gene expression (Fig. 1b). PPARGC1B in turn is responsible for constitutive non-adrenergic-mediated mitochondrial biogenesis via increased basal oxygen consumption [25], fat oxidation, non-oxidative glucose metabolism, and regulation of energy expenditure [25]. A pathway of biochemical events that seems to be confirmed here by the increase seen in HIF1 levels (Fig. 1c) and the corresponding overexpression of the VEGF gene family (Fig. 1c).

Additional markers of “mitochondrial health” showed significant changes. The mitochondrial transcription factor A (TFAM) that encodes a protein critical in both mitochondrial DNA repair and replication was higher in LAM than in control SMCs (Fig. 2a) and has previously [25] been linked to PPARGC1B gene expression (Fig. 1b). PPARGC1B in turn is responsible for constitutive non-adrenergic-mediated mitochondrial biogenesis via increased basal oxygen consumption [25], fat oxidation, non-oxidative glucose metabolism, and regulation of energy expenditure [25]. A pathway of biochemical events that seems to be confirmed here by the increase seen in HIF1 levels (Fig. 1c) and the corresponding overexpression of the VEGF gene family (Fig. 1c).

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Materials and methods

Lung tissue samples were obtained from human lung transplant donors, in accordance with the Declaration of Helsinki and approved by the Institutional Review Board at the University of Pennsylvania [5]. Four patient derived cell lines LAM-100, LAM-111C, LAM-D9065, and LAM-HUP were used in the present study. Controls were primary,
normal human bronchial SMCs (Lonza, Basel, Switzerland). Electron microscopy on 90-nm-thick sections were performed using a Jeol 1200 and Jeol 1400 transmission electron microscope (Jeol Ltd, Tokyo, Japan) at 80 kV. Images were acquired using an integrated MegaView III digital camera (Olympus Soft Imaging Solutions GmbH, Munster, Germany). Flow cytometry was performed on Rhodamine-123 (RH-123) (Sigma-Aldrich, St Louis, MO, USA).
USA)-treated cell suspensions using a FACS Canto II flow cytometer (BD Immunocytometry Systems, Erembodegen, Belgium). Fluorescence microscopy images were acquired by an Olympus IX-81 (OLYMPUS Corporation, Tokyo, Japan) light and fluorescent microscope. RNA was isolated with MN Nucleospin RNA isolation kit (Macherey-Nagel, Düren, Germany). RNA concentration was measured using NanoDrop (Thermo Fisher Scientific, Waltham, USA). TaqMan PCR reaction was performed using ABI StepOnePlus system and data were analyzed with StepOne software. MicroRNA expression was normalized to U6 expression. Nanostring assay was analyzed using the nCounter Analysis System (NanoString Technologies, Washington, USA). Angiogenesis was assessed using a Human Angiogenesis Array Kit (R&D Systems, Minneapolis, USA). Protein concentration was determined using a fluorescent protein assay (Qubit protein; Thermo Fisher Scientific, Waltham, USA). Quantitative RT-PCR was performed using SensiFAST SYBR Green reagent (BioLine, London, UK) in an ABI StepOnePlus system (Thermo Fisher Scientific, Waltham, USA) and data were analyzed with StepOne software and normalized to beta-actin as a housekeeping gene and calculated according to the 2−ΔΔCt method. Array data were evaluated using a feedforward artificial neural network (ANN) (Neurosolutions 6; NeuroDimension Inc.) software. Metabolic profiling was performed using Seahorse XF96 (Agilent Technologies, USA) [11] and Oroboros (O2k, OROBOROS Instruments, Innsbruck, Austria) platforms [12]. Transwells were used for migration assay (Costar, Corning Incorporated, Sigma-Aldrich, St Louis, MO, USA). TRXR activity was assessed using a Thioredoxin Reductase Assay Kit (Abcam, Cambridge, MA, USA). Statistical analysis was performed using the independent samples t-test and one-way ANOVA with Bonferroni correction. P<0.05 was considered as significant. For extensively detailed Materials and methods refer to Full Methods (Supplementary Material).

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Author contributions EMMA: performed the experiments, isolated RNA and protein from SMC and LAM, cellular staining, arrays, nanostring, embedding of samples for microscopy, performed data analysis, prepared figures; RR and KV generated the LAM cell lines; SP performed ANN analysis; PB performed Seahorse analysis; VS performed clinical overview; KK performed Nanostring analysis; DMcP developed Proxison; JEP designed the studies; EMMA, KK, VS, DMcP, AP, and JEP have written the manuscript.

Compliance with ethical standards

Conflict of interest DMcP and AP own shares/employed by Antoxis Ltd, UK. The remaining authors declare that they have no conflict of interest.

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