MOLECULAR REGULATION OF LYMPHANGIOLEIOMYOMATOSIS

PhD thesis

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Introduction

Lymphangioleiomyomatosis (LAM) is a rare, slowly progressing, fatal systemic disease that affects mainly young women. LAM is defined by the overgrowth of atypical smooth muscle (SMC)-like cells known as LAM cells in the lungs, lymphatics and soft organs. Excess growth of atypical SMC-like cells leads to cyst formation within the lungs resulting in pneumothoraces and lung collapse. LAM was formerly considered as a benign disease, but recently such cyst forming cellular growth has been reclassified as slow-growth-rate metastasizing neoplasm. The suffering of LAM patients increases gradually as the disease progresses. LAM is categorized as a rare disease worldwide with an incidence rate of 1-9/1 000 000. In Europe, numbers are much higher where the sporadic form of LAM affects 1/500,000-1/125,000 women. Also, LAM characteristic mutation of the tuberous sclerosis (TSC) gene is present in 1/6000 births in Europe. 30-40% of people carrying TSC mutation develop LAM in their adulthood. With the new and more sensitive diagnostic methods, numbers are climbing worldwide and increase significantly in Europe. The majority (86%) of patients need lung transplantation within 10 years after diagnosis.

Most of LAM patients present to the clinic with shortness of breath, chest tightness, collection of air in the pleural space (pneumothorax) and excess fluid between the layers of the pleura (pleural effusion). These symptoms are not specific to LAM, therefore the disease is often misdiagnosed as asthma or chronic obstructive pulmonary disease (COPD). This misdiagnosis leads to failed treatment and faster disease progression as asthma and COPD medications have limited effects on the symptoms. Diagnosis can only be made if vascular endothelial growth factor D (VEGF-D) (above 800 pg/ml) and matrix metalloproteinase (MMP) plasma levels are measured and lung CT scan confirms the diagnosis.

LAM is caused by inherited (TSC-LAM) or acquired (sporadic or S-LAM) mutations of the tumour suppressor tuberous sclerosis complex (TSC) genes TSC1 (hamartin) or TSC2 (tuberin). The TSC1-TSC2 complex interacts with various signalling 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. 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 de-regulation of signalling pathways *via* an alternative route which possibilities are actively researched in leading laboratories of the field. The origin of characteristic LAM cells is still

unknown, despite the indicative presence of various tissue markers.

Reactivity to α-SMA and HMB45, altered mTOR-S6P activity, and TSC1/2 mutations are all characteristic to LAM cells. Expression of the melanocytic marker HMB45 has raised a theory that LAM cells might potentially develop in the neural crest. Certainly, cyst formation in tissues that originate from the neural crest lineage support such theory. Another hypothesis places the uterus as the origin of LAM cells. This theory is supported by the high levels of ER and PR expression in LAM cells showing 30 % correlation with angiomyolipomas, perivascular epithelioid cell tumours (PEComas) and mesenchymal tumours most commonly found in the uterus. Unfortunately, even if the origin of the LAM cells is going to be resolved, the theory could not explain how the cyst migrates and metastasizes to the lung.

When mTOR is activated caused by TSC1/2 mutation, *two complexes* can form: mTORC1 or/and mTORC2. mTORC1 or/and mTORC2T positively regulates cell growth, proliferation, autophagy and mitochondrial metabolism and biogenesis. Currently, treatment options remain limited to the mTOR inhibitor Rapamycin (Sirolimus) 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 a "crude" alternative route to preventing disease progression. Although such attempts have mostly failed, we theorized that it was not the original idea but the study approach that might have been unsuccessful.

The mTOR pathway has many up-stream regulatory molecules and pathways including growth factors, energy depletion mechanisms, changes in oxygen levels, amino acids (leucine and arginine), altered levels of inflammatory cytokines, estrogen etc. One of the main mTORC1 regulatory pathways is the evolutionarily conserved Wnt signalling pathway. Currently, the regulatory factors of the mTOR pathway are under intense investigation as they might provide novel and potentially more effective therapeutic targets.

Aims

In the present research it was the primary aim to understand the molecular background and the broader mechanisms (in addition to TSC mutations) of LAM in more detail which understanding can lead to potential identification of novel therapeutic targets.

The aims can be grouped into three main areas:

1- To investigate the underlying hormone dependency of LAM disease.
2- To investigate the role of Wnt signalling in the modulation of LAM disease development and progression.
3- To test novel drugs that have shown characteristic efficacy in other characteristically female diseases (e.g. breast cancer).

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. 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 smooth muscle cells (SMC) (Lonza, Basel, Switzerland). **Electron microscopy** on 90 nm thick sections were performed using 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) 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). **Human Nuclear Receptors TaqMan®Array** and **Human Wnt Signalling TaqMan®Array** (Thermo Fisher Scientific, Waltham, USA). TaqMan PCR reaction was performed using ABI StepOnePlus system and data were analysed with StepOne software. MicroRNA expression was normalized to U6 expression. **Nanostring assay** was analysed 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^{-ddCt} method. Array data was evaluated using a feed forward **artificial neural network (ANN)** (Neurosolutions 6, NeuroDimension Inc.) software. **Metabolic profiling** was performed using *SeaHorse XF96* (Agilent Technologies, USA) and *Oroboros* (O2k, OROBOROS Instruments, Innsbruck, Austria) platforms. 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.

RESULTS

Characterisation of LAM cell lines

LAM cell lines (LAM100, 111c, D9065, HUP) used in the study were isolated from four LAM patients undergoing lung transplantation in Philadelphia, USA (National Disease Research Interchange (NDRI), Philadelphia, PA, USA). LAM cell lines carrying TSC2 mutations demonstrated hyperactive mTORC1 signalling in approximately 76% of cells and demonstrated high proliferation rate, increased migration and invasiveness, characteristic to neoplastic cell lines, compared to the same passage number of primary human lung fibroblasts during cell characterisation (Dr Krymskaya (Perelman School of Medicine, University of Pennsylvania, USA). Two normal SMC cell lines were isolated from healthy lung donors and were purchased from Lonza. SMCs were tested for *a*-SMA and von Willebrand Factor (Factor VIII) by the manufacturer. To compare the normal and diseased LAM cell lines, haematoxylin eosin and *a*-SMA IF staining was performed. Using the above techniques, no significant differences were detected between LAM cells and SMCs.

To characterise the molecular differences, qRT-PCRs of HMB-45, mTOR, TSC2 and PS6 genes were performed and confirmed with immunofluorescent staining. Significant up regulation ($P<0.05$) of mTOR (originally reported by Dr Krymskaya et al) and PS6 genes in LAM cell lines were detected compared to normal SMCs controls. Also, a total loss of TSC2 gene expression was observed in LAM cells. As both TSC1/2 genes are essential for regulation of the mTOR pathway, the observation that the TSC2 gene produced no transcript was an important factor to use these patient derived cell lines as models of LAM disease.

LAM molecular background mapping: Deregulation of nuclear receptors, vascularization and miRNAs

Estrogen hormone affects cellular signalling 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). 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. Of the 92 examined genes, 21 have shown an increase, and a further 30 a reduction in expression levels when compared to normal bronchial SMCs. The nuclear receptor TaqMan array and consequent qRT-PCR analysis on individual cell types identified the progesterone receptor (PGR), the peroxisome proliferator-activated receptor gamma coactivator 1-beta (PPARGC1B) as being significantly overexpressed ($P<0.001$) and estrogen related receptor gamma (ESRRG) as markedly upregulated ($P<0.05$). 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. PPARGC1B overexpression is linked to increased mitochondrial number, the active PRG isoform 4 to increased mitochondrial membrane potential and cellular respiration, while ESRRG to control of mitochondrial biogenesis and energy metabolism. The significantly down-regulated nuclear receptor genes included NR5A2 and retinoic acid receptor beta (RARB). RARB is a member of the thyroid-steroid hormone receptor superfamily and both genes are known to play a powerful role in the inhibition of proliferation and stimulation of cellular differentiation. ANN analysis of the data sets revealed a strong positive correlation with several retinoic acid receptors (RARB, RXRB, RXRG) all of which bind the biologically active form of vitamin A and PGR. Changes in nuclear receptor gene expression in the disease cell lines directly point towards a 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. 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). Such changes in energy metabolism inevitably lead to an increased expression of the hypoxia-inducible factor 1 alpha (HIF1-alpha) ($P<0.05$), and consequently to increase in VEGF expression. In support, qRT-PCR analysis and an angiogenesis protein array detected strong up-regulation of LAM diagnostic markers VEGFC and VEGFD ($P<0.001$).A decrease in thrombospondin-1 (TSP1) and an increase in CXCL16 chemokine peptide levels was also observed in the LAM samples.

These observations are particularly interesting as TSP1 is a recognized inhibitor of mitochondrial biogenesis, while CXCL16 regulates cellular invasion in non-small cell lung cancer (NSCLC). ANN analysis of the angiogenesis array data identified a strong association of CXCL16 and TSP1 with the fibroblast growth factor (FGF), Endothelin1, SerpinE1 and VEGFC levels that are all involved in the stimulation of vascularization and the induction of myofibroblastic phenotype that in itself can reduce the capacity for pulmonary regeneration.

Further studies using a Nanostring chip methodology identified down-regulation of both the tumour suppressor miR125b-5p and the low density lipid oxidation induced autophagy regulator miR155-5p in LAM. The apoptosis inducer miR-15b-5p was also down-regulated, while the cell proliferation and survival inducer miR-199a/b-3p had increased copy numbers in individual LAM samples.

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

Mitochondrial dysfunction in LAM

Electron microscopic images of the normal bronchial SMC and LAM cell lines showed a drastically different mitochondrial morphology. Mitochondria in LAM cells were smaller, darker and so electro-dense that the inner membrane cristae were not visible. The gene profiling data again supports 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 several fold higher in LAM than in control bronchial SMCs and has previously been linked to PPARGC1B gene expression. PPARGC1B in turn is responsible for constitutive non-adrenergic-mediated mitochondrial biogenesis *via* increased basal oxygen consumption, fat oxidation and non-oxidative glucose metabolism, as well as the regulation of energy expenditure. A pathway of biochemical events that seems to be confirmed here by the increase seen in HIF1 levels and the corresponding over expression of the VEGF gene family.

Additional markers of "mitochondrial health" also showed significant changes. The mitochondrial transcription factor A (TFAM), that encodes a protein critical in both mitochondrial DNA repair and replication, was higher in the diseased LAM cell lines than in normal bronchial SMC controls. The observed alterations in transcription levels appeared to impact on all aspects of mitochondrial function. Both Cyto C (cytochrome complex), an inner

membrane protein of the mitochondria that is an essential component of the electron transport chain, as well as Cox4 (Cytochrome c oxidase), that catalyses oxygen reduction were significantly elevated.

To investigate overall mitochondrial activity in more detail, a combination of flow cytometric analysis of the mitochondrial membrane Rhodamine-123 (RH-123) a cell-permeable, cationic, green-fluorescent dye, as well as Oroboros and Seahorse analyses were performed. Mitochondrial energization induces quenching of RH-123 fluorescence and the rate of fluorescence decay is proportional to mitochondrial membrane potential. Based on this analysis, LAM mitochondrial membrane activity is twice as high as that seen in normal, bronchial SMC controls. In contrast, increased glycolysis and reduced oxidation was detected by metabolic analysis of LAM cells using Seahorse and Oroboros technology. At first sight, these biochemical results may appear contradictory, however, during reductive stress, when electron acceptors are expected to be mostly reduced, some redox proteins can donate electrons to O_2 instead. This process can increase net mitochondrial reactive oxygen species (ROS) production, despite the concomitant enhancement of ROS scavenging systems. For example, normally antioxidant matrix NADPH reductases, together with glutathione reductases and thioredoxin reductases (TrxR), can all go on to generate H_2O_2 by leaking electrons from their reduced flavoprotein to O_2. Generation of this net mitochondrial ROS spill-over can cause oxidative injury and can critically damage mitochondria. The process by which cells remove these damaged or dysfunctional mitochondria is known as mitophagy. Any damage to the mitophagy process may result in abnormal mitochondrial function. To test this theory, TrxR activity was determined in both normal, bronchial SMCs and LAM cell lines. In the latter, TrxR activity was significantly higher than in normal SMC controls.

Interestingly, the Trx2-TrxR2 system has been reported to be an anti-angiogenic target of auranofin, a redox enzyme inhibitor gold complex. The high affinity of auranofin for thiol and selenol groups and through the inhibition of redox enzymes such as TrxR, can modify the redox balance in mitochondria. Our studies reported here, together with the above supporting evidence from the published literature, we theorized that drugs that can inhibit TrxR activity and restore normal mitochondrial function, might be able to reduce LAM progression.

Mitochondria as potential therapeutic target in LAM

The novel synthetic flavonoid, Proxison (7-decyl-3-hydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone) (Antoxis Ltd, UK), is a potent antioxidant accessing the mitochondria. Proxison combines key structural attributes of the natural flavonoid myricetin, with a strategically placed lipophilic chain to effectively protect cell membranes from lipid peroxidation. To test the effects of Proxison, both normal bronchial SMCs and LAM cell lines were treated with the drug. Mitochondrial activity was measured using RH-123 and TrxR activity. Both tests showed the striking normalization of mitochondrial function in LAM cell lines while Proxison appeared to have had little or no effect on normal bronchial SMCs. Rapid improvement was detected in the mitochondrial morphology of LAM cells with cristae of the inner membrane becoming visible again by electron microscopy. Morphological changes were associated with the reduced gene expression of CytoC, NRF1, TFAM and Cox4 as well as with gene expression of VEGF ligands and receptors falling back to normal levels. Additional functional studies, scratch and migration assays have shown that Proxison treatment reduced the proliferation and migration capacity of LAM cells and such effect was additive to Rapamycin treatment in both gene expression and cellular migration.

Retinoic acid receptor as a potential therapeutic target in LAM

As the TaqMan array analysis detected reduction in RARβ expression, deregulation of vitamin A metabolism in LAM was presumed. qRT-PCR confirmed the initial findings as RARβ mRNA levels were reduced in individual LAM samples. To investigate the cause of RARβ downregulation, LAM cells were treated with retinoic acid (RA). Incubation of LAM cell lines with 2 μM RA restored RARβ levels to normal within 24 h. As RA restored RARβ gene expression to normal, it suggested the existence of a different fault. To decipher the mechanism, the overall vitamin A metabolism was analysed. There are two main sources of vitamin A, retinol, from animal source and β-caroten from plant source. One of the 600 known naturally occurring plant derived carotenoids is Lutein. The oxidised form of retinol is RA. Both sources have their own metabolic pathways to reach their shared nuclear receptors, and they also share some metabolic enzymes.

To study the reasons behind for RARβ deregulation, qRT-PCR was performed to test the relative quantity of enzymes involved in vitamin A metabolism. Alcohol dehydrogenase (ALDH1A1), β-carotene dehydrogenase 1 (BCO1) and β-carotene dehydrogenase 2 (BCO2) were significantly downregulated ($P<0.05$) in LAM cell lines compared to levels measured in normal SMCs. Practically, LAM cells were lacking BCO2 and ALDH1. Meanwhile, retinol dehydrogenase (RDH) was significantly upregulated in LAM cell lines. To investigate whether vitamin A metabolites affect enzyme levels, LAM cell lines and SMC controls were treated with RA and lutein. In contrast, Rapamycin induced a slight increase of BCO1 and RDH1 but had no effect on BCO2 and ALDH1 in LAM. Interestingly, while RA and lutein treatment restored the above enzymes close to normal levels, combination treatment with Rapamycin resulted in extreme increase in RDH1 and a complete loss of ALDH1 levels. All Rapamycin, RA and lutein increased RARβ levels to normal or above but a combination treatment with Rapamycin and lutein induced a five-fold increase in RARβ expression in control SMCs.

Scratch assay was used to assess the effect of RA treatment on cellular proliferation and migration capacity of LAM cells. Combination of RA with Rapamycin resulted in lower proliferation and migration capacity, than detected in Rapamycin monotherapy.

Deregulation of WNT signalling in LAM

As one of the more robust regulators of the mTOR pathway is Wnt signalling, individual human Wnt signalling TaqMan array plates were used to analyse the gene expression levels of molecules in the various Wnt signalling pathways. All four LAM patient derived cell lines and the two normal SMC cell lines were tested. Out of the investigated 92 genes, 43 genes were deregulated in LAM. 36 genes were significantly downregulated and 7 genes were upregulated. The downregulated genes included secreted extracellular inhibitors SFRP2, SFRP4 and DKK2. Earlier studies indicated inhibitory effect of SFRP4 on AMPK signalling pathway and mitochondrial depolarization. Also SFRP2 is regulated by AKT. Based on the literature, loss of SFRP4 leads to aggressive cancers, epithelial mesenchymal transition (EMT), increased cell migration and deregulation of downstream signalling molecules within the Wnt signalling pathways. Downregulation of DKK2 that inhibits STAT5 signalling can also have severe consequences. STAT5 proteins are activated by a wide variety of hematopoietic and non-hematopoietic cytokines and growth factors, and critically regulate vital cellular functions such as proliferation, differentiation, and survival. The physiological importance of active STAT5 proteins is obvious in a large number of primary human tumours that have aberrant constitutive activation of these proteins, which significantly contributes to tumor cell survival and malignant progression of the disease. DKK1, another extracellular inhibitor of the canonical Wnt pathway, however, was significantly upregulated, and based on the literature, DKK1 is known to promote migration and invasion in liver cancer via shifting canonical Wnt activation towards non-canonical Wnt pathway activity leading to increased inflammatory processes by activation of the NF-kB pathway. DKK1 has just recently been recognised to play an important part in the pathophysiology of arterial wall. The upregulated Wnt5b, one of the 19 ligands of the Wnt signalling pathway, is known for its role in activation of PPARγ and induction of adipogenesis as well as induction of tube formation by regulating the expression of Snail and Slug proteins via activation of both canonical and non-canonical Wnt signalling pathways. Wnt5b can also modulate mitochondrial activity via MCL1 and is known to promote cell motility and metastasis in various cancers. The intracellular canonical Wnt signalling mediator, GSK3β - an important regulator of the mTOR pathway - was also upregulated. Simple upregulation of GSK3β is not informative as its enzymatic activity strongly depends on its level of phosphorylation.

Deregulated activity of GSK3β has been observed in many human pathologies e.g. cancers and non-insulin-dependent diabetes mellitus (NIDDM). Phosphorylation of GSK3β can suppress its activity leading to β-catenin accumulation in the cytosol. The accumulated β-catenin molecules then translocate to the nucleus and increase canonical Wnt target gene transcription. Suppression of GSK3β by AKT and mTOR pathway can shift Wnt signalling to increased non-canonical Wnt pathway activity that has also been associated with cancer progression. Additionally, RHOU - a molecule that is involved in various cellular processes - was also upregulated. In *in vitro* experiments RHOU has oncogenic activity and promotes cancer cell invasion. Its increased expression and activity correlates with carcinogenesis in a variety of cancers.

CONCLUSIONS

The rare disease LAM, presents a largely unmet medical need. Although recently animal models have started to emerge, fully human models of the disease are important to fully reveal the molecular background and to identify therapeutic targets. In the present work a great variety of molecular platforms were used to study LAM in patient derived cellular systems.

Based on the study, it was identified that uncontrolled proliferation of SMC-like LAM cells is just partly the consequence of TSC mutation and deregulation of the mTOR signalling pathway. Mitochondrial dysfunction, deregulated vitamin A metabolism and altered Wnt signalling might also contribute to the disease.

Mitochondria are regulated via complex molecular pathways and in the absence of further mutation analysis it is still unclear whether simply TSC mutation can lead to mitochondrial deregulation and metabolic malfunction. However, as in about 15% of all LAM cases have no TSC mutations, one can speculate that mutations in mitochondrial or Wnt signalling pathway genes can also lead to similar symptoms. The present study identified many deregulated molecules including nuclear receptors of hormones such as estrogen, transcriptional coactivators such as PPARGC1α and PPARGC1β that together with peroxisome proliferator-activating receptor (PPAR) genes, estrogen-related receptor (ERR) genes and NRF-1 coordinate the energy metabolism. The altered expression of the above genes substantiated the importance of investigating the role of mitochondria in LAM pathogenesis. To make the picture even more complex, regulators of vitamin A metabolism and the Wnt signalling pathway is also deregulated in LAM and provided further targets for studies of therapeutic intervention.

Based on the results, restoration of mitochondrial activity is a strong candidate in LAM therapy. A pre-clinical drug candidate, Proxison, was chosen for the test. With its very high antioxidative capacity and capabilities to normalize both the electron transport chain and membrane potential, Proxison appeared to be a “wonder drug” for correction of mitochondrial dysfunction. Many flavonoids - such as myricetin and quercetin - are known for their antioxidative activity specifically targeting mitochondria, but Proxison’s modified structure

improved its antioxidative ability as became superior in healing diseased mitochondria.
True to expectations, Proxison restored mitochondrial activity and corrected deregulated gene expressions while was no cytotoxic to cells. In fact, Proxison had more robust effect on cell functions than Rapamycin, the clinically approved drug for LAM treatment.

Molecular mapping of patient derived cell lines have also revealed deregulation of additional pathways that interact with mTOR. Previous studies have found that RAR and estrogen oppose the action of each other. Furthermore that estrogen response element (ERE) and retinoic acid response element (RARE) colocalize in the genome and regulate the expression of shared target genes. It was suggested that ER and RAR may compete for transcriptional activity or inhibit each other depending on the availability of their ligands. Since ERRG also binds to ERE, increase in its transcriptional activity may decrease the expression of RARs. RA competes with estrogen to inhibit or trigger proliferation, respectively. RA is an antitumor agent known to be anti-proliferative, pro-apoptotic and anti-metastatic. Treatment of LAM cell lines with RA restored normal levels of RARβ within 24 h of treatment. RA not just restored RARβ mRNA expression to normal levels but also lead to reduced proliferation as well as cellular migration of LAM cells. Combination of RA with Rapamycin, however, have also raised some warning signs as gene expression of vitamin A metabolic enzymes were markedly deregulated in combination treatment.

Molecular mapping of the evolutionarily conserved Wnt signalling pathway has also identified some potential therapeutic targets. Deregulation of canonical Wnt pathway inhibitors DKK1 and DKK2, SFRP 2 and 4 are all associated with aggressive carcinogenesis and recently became therapeutic targets not just in carcinomas but in other diseases associated with inflammation and vascularization. The upregulated ligand, Wnt5b and the signalling molecule GSK3β are also involved in mTOR signalling but further studies are needed to understand their specific roles in LAM development and progression.

Some factors of various signalling pathways and especially mitochondrial dysfunction that was exposed during the study can be used as potential therapeutic target in LAM. The effects of both Proxison and RA were particularly promising to reverse mitochondrial dysfunction and to halt uncontrolled proliferation, respectively. As combination of Proxison and/or RA with Rapamycin could even decrease the clinically applied dosage of Rapamycin, the current results could almost immediately have a direct effect on Rapamycin dependency of LAM

patients leading to reduced severity of Rapamycin side effects.

SUMMARY OF NOVEL FINDINGS

The study investigated the molecular background of LAM disease. The investigation led to the identification of the general deregulation of various nuclear receptors, molecules in vascularization and their miRNA regulators. The summary of these finding pointed toward mitochondrial malfunction.

Detailed study of the mitochondria using SeaHorse, Oroboros and electron microscopy confirmed mitochondrial dysfunction in LAM cells.

Proxison, a pre-clinical drug candidate with a very potent antioxidative capacity restored mitochondrial activity and corrected deregulated gene expressions implicating mitochondria as a novel target in LAM therapy.

Molecular mapping has also revealed deregulation in retinoic acid receptors. RARβ is a target for both estrogen and retinoic acid which regulate cell proliferation. Retinoic acid treatment restored normal levels of RARβ on mRNA level and also reduced proliferation rate in LAM cell lines. Data highlighted vitamin A metabolism as an additional therapeutic target in LAM.

Finally, Wnt signalling pathways have also been deregulated in LAM. Identification of specific signalling molecules might also provide further therapeutic targets for treatment of LAM sufferers.

LIST OF PUBLICATIONS

Total impact factor: 14.541

The thesis is based on the following publications:

Published work:

Abdelwahab EMM, Pal S, Kvell K, Sarosi, V, Bai, P, Rue, R, Krymskaya, V, McPhail, D, Porter, A, Pongracz, JE: Mitochondrial dysfunction is a key determinant of the rare disease lymphangioleiomyomatosis and provides a novel therapeutic target. *Oncogene* 2018:1. doi:10.1038/s41388-018-0625-1 (IF: 6.854)

Manuscript in preparation:

Abdelwahab EMM, Bovari-Biri, J, Sarosi, V, Fillinger, J, Harko, T, Moldvay, J, Krymskaya, V, McPhail, D, Porter, A, Pongracz, JE: Vitamin A metabolites normalize metabolic enzyme expressions and decrease neoplastic characteristics of LAM cells.
2019. British Journal of Pharmacology

Other publication:

Published work:

Abdelwahab, E.M.M., Rapp, J., Feller, D., Csongei, V., Pal, S., Bartis, D., et al. (2019). Wnt signaling regulates trans-differentiation of stem cell like type 2 alveolar epithelial cells to type 1 epithelial cells. Respir. Res. 20: 204. (IF:3.829)

Pénzes Á, **Abdelwahab EMM**, Rapp J, *et al*. Toxicology studies of primycin-sulphate using a three-dimensional (3D) in vitro human liver aggregate model. *Toxicol Lett* 2017;**281**:44–52. doi:10.1016/J.TOXLET.2017.09.005 (IF: 3.858)

Presentations:

Abdelwahab, Elhusseiny. Pal, S. Kvell, K. Sarosi, V. Bai, P. Rue, R. Krymskaya, V. McPhail, D. Porter, A. Pongracz, JE. Mitochondrial dysfunction is a key determinant of the rare disease Lymphangioleiomyomatosis A MAGYAR TÜDŐGYÓGYÁSZ TÁRSASÁG 2018.

Abdelwahab, Elhusseiny. Pal, S. Kvell, K. Sarosi, V. Bai, P. Rue, R. Krymskaya, V. McPhail, D. Porter, A. Pongracz, JE. **Mitochondrial dysfunction is a key determinant of the rare disease lymphangioleiomyomatosis and provides a novel therapeutic target** International Cholnoky symposium 2018.

Poster:

Abdelwahab, Elhusseiny. Pal, S. Kvell, K. Sarosi, V. Bai, P. Rue, R. Krymskaya, V. McPhail, D. Porter, A. Pongracz, JE. **ROLE OF MITOCHONDRIA IN LYMPHANGIOLYOMATOSIS.** Targeting Mitochondria Congress Berlin 2017