Self-Renewal Properties of LAM cells

Final Research Report for the LAM Trust of NZ

JW Baty and M Berridge
Malaghan Institute of Medical Research

Background

Lymphangioleiomyomatosis (LAM) is a progressive cellular disease characterized by excessive smooth muscle-like cells (LAM cells) in the lungs of women in their childbearing years. It occurs as an isolated disorder with a prevalence of approximately 1-5 per million women, and also occurs in up to 40% of patients with tuberous sclerosis complex (TSC), which is an autosomal disorder affecting 1/5,800 [1-3]. Involvement of tumour suppressor genes (TSC1 or TSC2), uncontrolled cellular proliferation, abnormal smooth muscle cell differentiation and disease progression, suggests that LAM is a cancer-like disease in which regulation of normal cell growth has been lost.

LAM cells

LAM cells invade lung tissue forming nodules and causing cystic destruction and remodeling of the lung interstitium as well as obstructing airways and pulmonary lymphatics, and leading to the development of cystic structures [1,2,4]. Within the nodules that line the airways and cysts there are at least two LAM cell subpopulations: the nodule centres are predominantly made up of thin myofibroblast-like spindle-shaped cells which highly express Proliferating Cell Nuclear Antigen (PCNA), a marker of cell proliferation; while the periphery of the nodules are comprised of larger epitheloid-like cells which have low PCNA but higher expression of gp100, a glycoprotein that is recognized by the HMB-45 antibody and is usually expressed by melanoma cells and immature melanocytes [5,6]. LAM cells also express estrogen and progesterone receptors, which may explain the sex and age specificity of the disease [6]. LAM cells have metastatic potential and are able to spread to or from the lungs and pulmonary lymphatics resulting in the formation of renal or abdominal angiomyolipomas [7,8]. LAM cells can also invade new lung tissue after transplantation [9].

Cancer stem cells and self-renewal

The similarities of LAM with cancer raise the possibility that there might be a hierarchy of LAM cells with differential tumourigenic potential similar to that proposed in the cancer stem cell model (Figure 1).

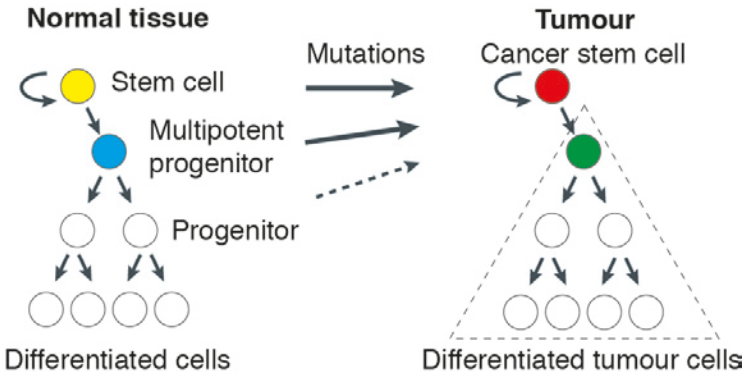


Figure 1. Cancer stem cell model. In normal tissue, undifferentiated stem cells (shown in yellow) can undergo division to reform stem cells (self-renewal) or to form multipotent progenitor cells (in blue). Multipotent progenitor cells undergo further division and differentiation to fill biological niches. Similarly, in the cancer stem cell model, cancer stem cells (in red) can self renew and form differentiated tumour cells (diagram modified from Zhou *et al.* 2009 [10]).

Cancer stem cells can been described as *the subpopulation of cancer cells with stem cell-like ability to produce all cancer cell types found within a tumour*. They are typically undifferentiated cells that have the potential to differentiate into the various cell types that comprise the original tumour. The first goal of this project was to investigate the potential for LAM to be sustained by a population of cells with self-renewal properties as proposed in the cancer stem cell model. We hypothesized that the mixture of cell types in LAM lung nodules are undifferentiated and differentiated cells. Our specific questions were:

Do primary LAM cells have cancer stem cell properties?

I. Can early passage primary LAM cells form spheres in stem cell media?

II. Do LAM cells growing in stem cell media express self-renewal genes?

Results

Primary cells

Primary, early passage LAM (six samples) and non LAM smooth muscle cells (three samples) were obtained from Prof Judy Black and Dr Lyn Moir (Woolcock Institute, Sydney) and were shipped at passage 3-5. The LAM cell samples included a cell type from chylous fluid. We found that the primary cells could be subcultured out to a passage number of approximately 20-25 before they stopped dividing. Experiments were conducted with cells that were at passage number less than 12.

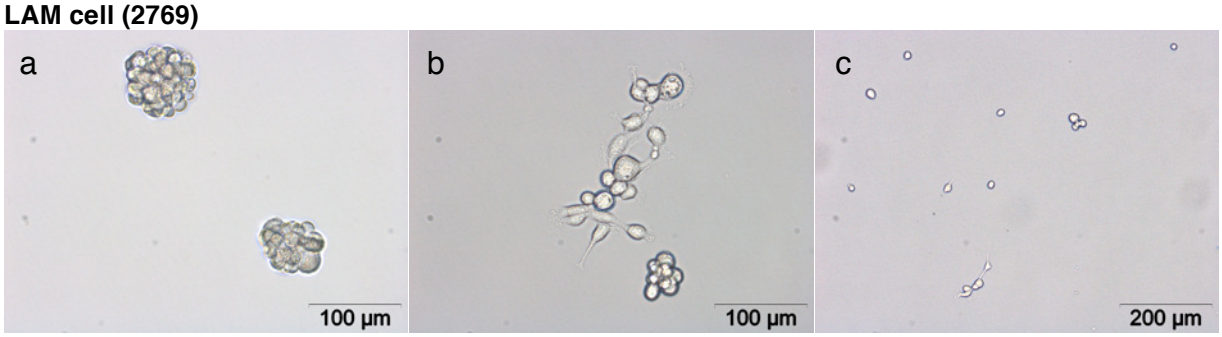
Self-renewal

(I) Sphere formation

One of the key characteristics of stem cells and cancer stem cells is self-renewal, which is the ability to form new stem cells. Self-renewal can be demonstrated by sphere formation in 'stem cell favouring media' and also by increased stem cell gene expression [11]. Spheres are non-adherent 3D formations of cells that arise by the division of a single cell. Combined with the increased expression of embryonic stem cell transcription factors (eg Sox2, Oct4 and Msi1), which can confer stem cell properties on cancer cells, the sphere formation assay indicates that the cells within spheres have characteristics of stem cells.

Primary cells were cultured in stem cell media for 2-4 weeks. However, multiple experiments indicated that the panel of LAM and non LAM lung cells were not able to form spheres typical of cancer stem cells. Instead, some cells formed non-adherent clusters of associated cells (Figure 2a and d), while others remained adherent (Figure 2b and e). Typically cancer stem cells readily form spheres even after disruption and re-passaging. However, when the LAM and non LAM cell clusters were disrupted and re-passaged, only single cells in suspension persisted (Figure 2c and f).

LAM cell (2769)



Non LAM cells (3303)

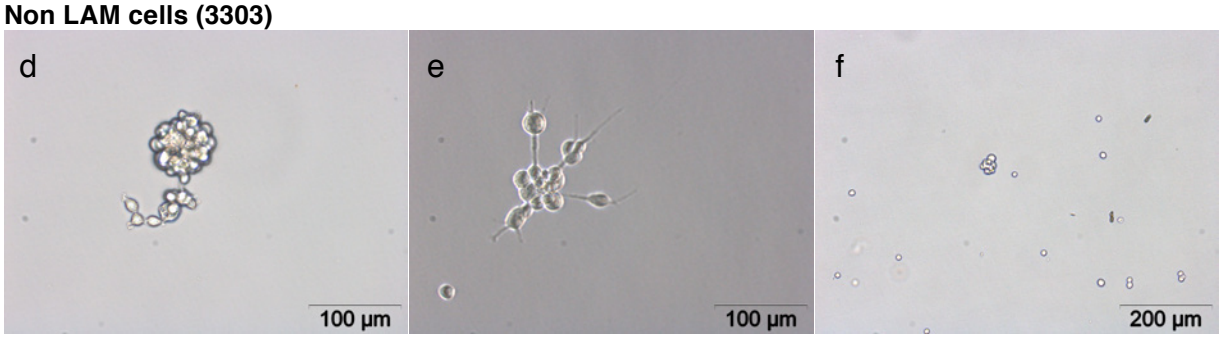


Figure 2. Examples of primary LAM (2769) and non-LAM (3303) cells grown in stem cell media. Non adherent cell clusters (a and d), adherent cell formations (b and e), and non adherent single cell suspensions observed after passaging (c and f).

(II) Self renewal genes

The primary LAM and non LAM cells that had been grown in stem cell media were analyzed for the expression of self-renewal genes associated with stem cell phenotype (Sox2, Oct 4, Msi1, BMPR2). Even though the cells were not able to form spheres, the expression of self-renewal genes was increased in both LAM and non LAM primary cells (Figure 3). The apparent increase in some stem cell transcription factors in individual LAM cell types compared with non LAM cells (e.g. Sox2 in LAM 2847 and LAM 2860; Oct4 in LAM 2860, and BMPR2 in LAM 2847) was not statistically significant for individual transcription factor comparisons between LAM and non LAM cells. However, for each gene tested, the highest values were seen in LAM cells.

It is unclear how upregulation of self-renewal genes relates to the inability of the cells to divide in stem cell media and it is possible that additional factors are required. These results suggest that upregulation of self-renewal genes is a general characteristic of lung smooth muscle cells grown under these conditions and not specific to LAM or non LAM cells.

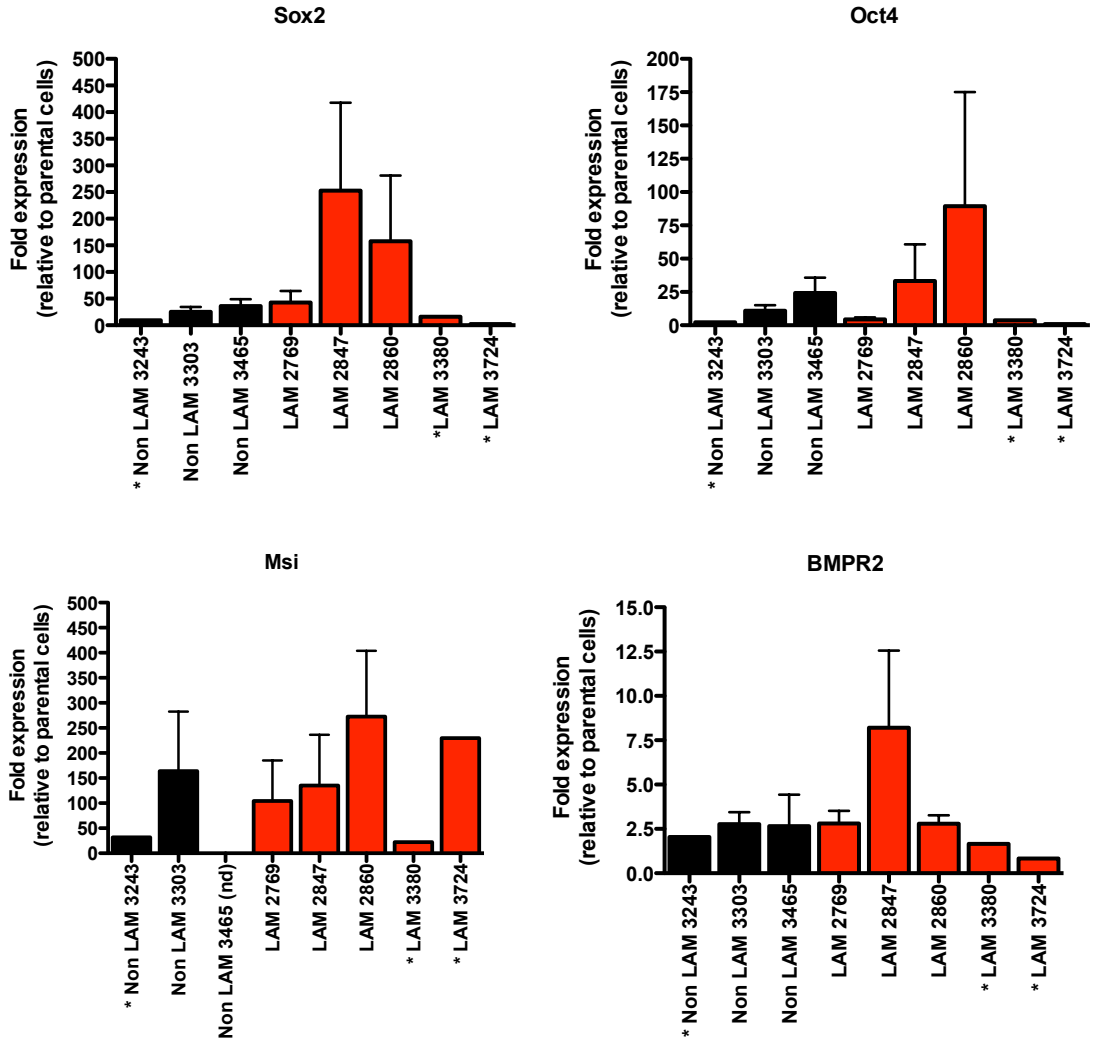


Figure 3. The expression of stem cell transcription factors and BMPR2 in primary LAM and non-LAM cells grown in stem cell media. Gene expression was measured by quantitative reverse transcriptase PCR. Samples indicated with an asterix (*) have only been tested once, the others have been tested at least twice and are represented by the mean and SEM.

Experiments to distinguish between LAM and non LAM cells

Approaches including staining with HMB-45 antibody, gp100 gene expression, estrogen receptor gene and protein expression, response to estradiol, preliminary treatment with human growth hormone, and sensitivity to rapamycin treatment failed to show any significant differences between the primary LAM and non LAM cells. We can think of at least two possibilities that may account for this: (1) the primary LAM cells have adapted to the culture conditions, perhaps with epigenetic changes that mask their abnormal phenotype; (2) non LAM smooth muscle cells have been present in the original LAM samples and have overgrown the LAM cells in culture. Both these scenarios would explain why LAM cells, which had been verified as HMB-45 positive in primary tissue and early passage cultures at the Woolcock Institute, were no longer HMB-45 positive in our

experiments. In the first scenario our hypothesis is that additional factors, possibly estrogen, are present in patients that contribute to the LAM cell phenotype. In cell culture, the absence of these factors causes the LAM cells to revert to a more normal phenotype.

TSC2-/- cells

In subsequent work, we focused our attention on TSC2 knockout cells (TSC2 -/-). TSC2 is a key intracellular signalling protein that is inactivated by a two-hit mutation in. Normal TSC2 forms a heterodimer with TSC1 that inhibits the activity of a central signalling hub known as mTOR complex 1 [12]. However, a loss of function mutation in TSC2 leads to the constitutive activation of mTOR complex 1 and results in the unregulated cell growth and abnormal phenotype observed in LAM. We were provided with TSC2 wildtype (+/+), and TSC2 knockout (-/-) mouse embryonic fibroblasts (MEFs) [13], as well as TSC2 -/- TMKOC cells from Assoc. Prof Vera Krymskaya, University of Pennsylvania. We looked at the effect of targeted drug treatment on the cells (rapamycin and metformin), and also how the cells responded to specific conditions of stress (serum deprivation, hydrogen peroxide, vitamin C, and hypoxia).

Drug treatment: Rapamycin and Metformin

Constitutive activation of the mTOR complex 1 due to inactivated TSC2 can be overcome by treatment with the immuno-suppressant drug rapamycin (also known as sirolimus). Rapamycin has been shown to restore homeostasis in cells with defective TSC gene function, and to be useful in treating LAM-related lung disease [14]. Whereas rapamycin is thought to specifically target mTOR complex 1, the diabetes drug, metformin, has multiple intracellular targets that include mTOR complex [15]. We treated the TSC2 +/+ and -/- cells with metformin and rapamycin to see if combining the drugs might be more effective. TSC2 -/- MEFs were more sensitive to rapamycin treatment than TSC2 +/+ cells even at low concentrations (2 nM) (Figure 4A). However, there was no difference between the TSC2 -/- and +/+ MEFs with metformin treatment and a relatively high concentration of metformin (10 mM) was needed to cause an approximately 50-60% decrease in metabolic activity (Figure 4B). A combination of the drugs was slightly more effective than either agent alone, but there was no specificity for TSC2 -/- MEFs. The combination of rapamycin with metformin did have a slightly greater effect but still did not cause the

complete loss of metabolic activity. These results indicate that this combination is unlikely to be a promising therapeutic option.

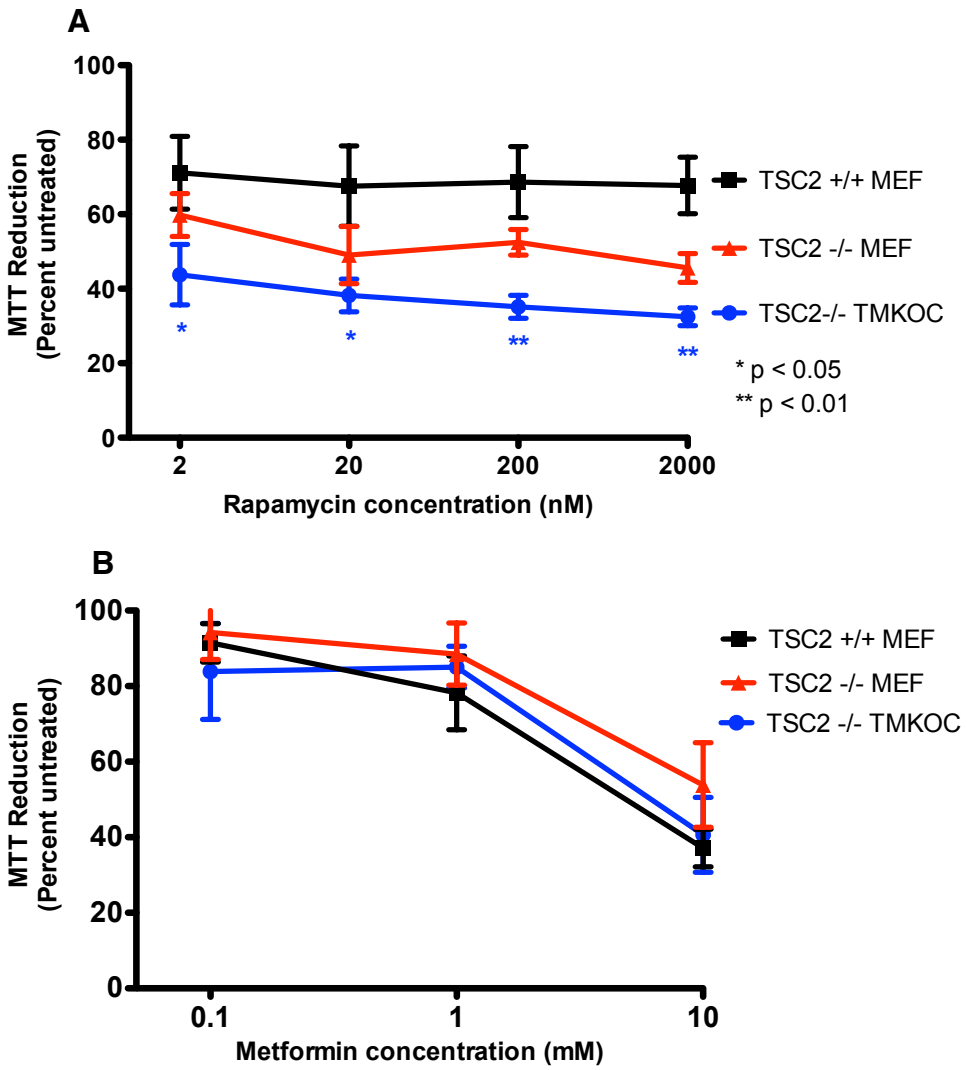


Figure 4. The effect of rapamycin (A) or metformin (B) on TSC2 +/+ and TSC2 -/- cells. Cells were treated with rapamycin or metformin for two days and their metabolic activity was measured using the MTT assay. Experiments were repeated three times.

Stress

The abnormal activation of mTORC1 activity gives LAM cells a growth advantage so that they are able to proliferate and cause lung damage in LAM patients. We investigated the possibility that TSC2 negative cells that have abnormal mTORC1 activity might show increased resistance to various stress conditions.

Serum deprivation

Cells grown *in vitro* require various supplements in order to survive. These include fetal calf serum (FCS), which is usually maintained at about 10% in the cell media. A defining characteristic of TSC2 -/- cells is their ability to continue proliferating in the absence of serum in culture [13]. We tested the metabolic activity of the panel of TSC2 +/+ and -/- cells grown in 10%, 1% and 0% FCS for 1 - 5 days (Figure 5). Serum deprivation had the greatest effect on the TSC2 +/+ cells, while the TSC2 -/- cells were relatively resistant.

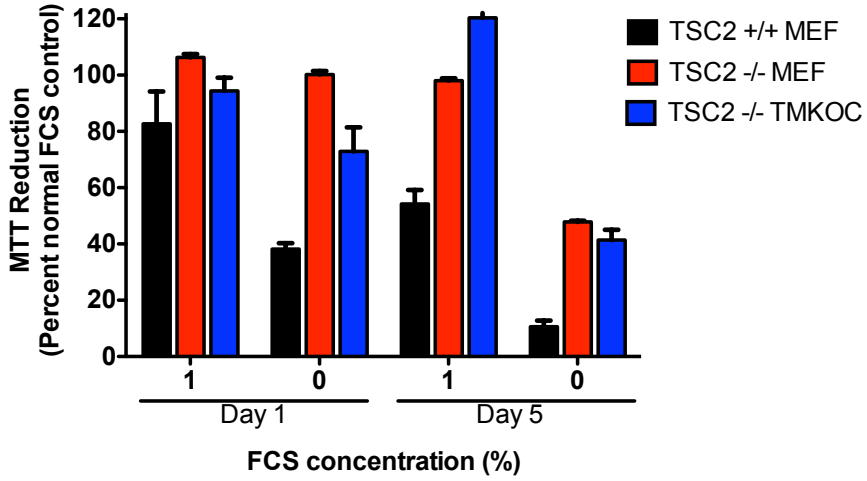


Figure 5. The effect of serum deprivation on TSC2 +/+ and TSC2 -/- cells.
Cells were grown for 1-5 days and their metabolic activity was measured using the MTT assay.

Hydrogen peroxide

To test our hypothesis that TSC2 -/- cells would also be resistant to other forms of stress such as oxidative stress we treated TSC2 +/+ and -/- cells with hydrogen peroxide and monitored their viability and proliferation. Hydrogen peroxide is an oxidant that can reach high levels at sites of inflammation and cause cellular damage. The panel of TSC2 +/+ and -/- cells were treated with a range of hydrogen peroxide concentrations for 24 hours

and the metabolic activity of the cells measured using MTT assay (Figure 6). The viability of the cells was also measured by flow cytometry (not shown). In all cases, high concentrations of hydrogen peroxide (900 μM) caused cell death or complete loss of metabolic activity, which was expected. However, the TSC2 -/- cells were significantly more resistant to the intermediate concentrations of hydrogen peroxide tested (700-800 μM) (Figure 6). These results raise the possibility that TSC2 -/- cells are better equipped to survive in the presence of oxidants than normal cells, conferring a growth advantage in sites of oxidative stress.

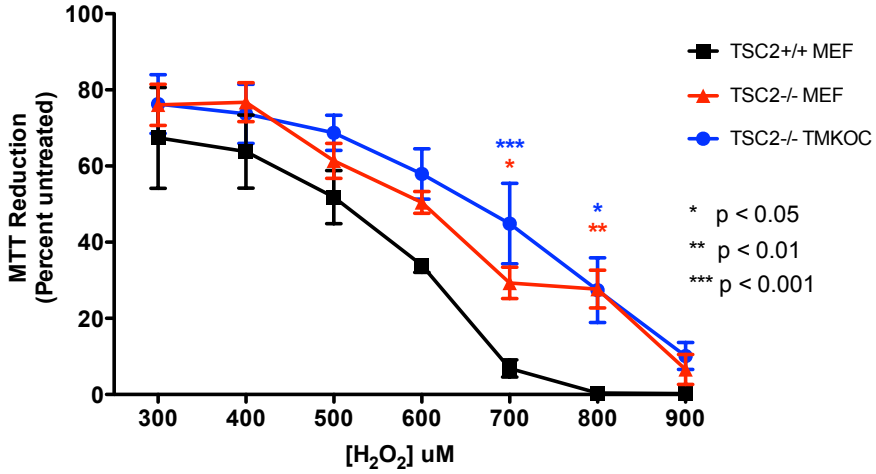


Figure 6. The effect of hydrogen peroxide on TSC2 +/+ and TSC2 -/- cells.
Cells were treated for 24 hours with hydrogen peroxide (H_2O_2) and their metabolic activity was measured using the MTT assay. The average and SEM of three separate experiments are shown.

Catalase

Catalase is a cellular enzyme that breaks hydrogen peroxide down to form water and oxygen. We conducted preliminary experiments to see if TSC2-/- cells had higher levels of catalase, which would explain their resistance to hydrogen peroxide. However, the TSC2-/- cells did not appear to have increased catalase levels over TSC2 +/+ cells.

High concentration vitamin C

We explored the effect of high concentration vitamin C on the cells. High concentrations of vitamin C have been found to be toxic to cells, particularly some cancer cell types, and this may be due to the vitamin C-initiated production of hydrogen peroxide [16]. The TSC2 +/+ and -/- cells were treated with vitamin C and then we measured their metabolic activity (Figure 7) and viability (not shown). Vitamin C concentrations greater than 2.5 mM caused a decrease metabolic activity of all the cells, and 5 mM treatment eradicated metabolic activity completely. However, our experiments did not show any significant differences between the TSC2 +/+ or -/- cells.

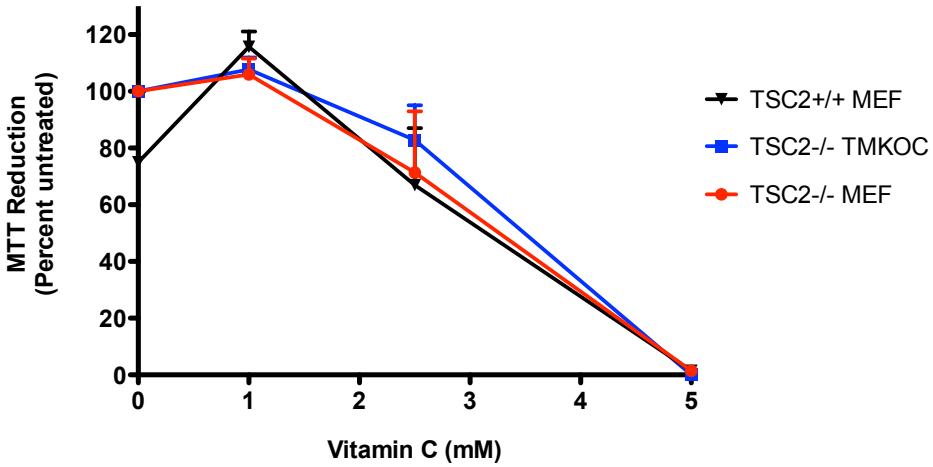


Figure 7. The effect of vitamin C on TSC2 +/+ and TSC2 -/- cells.
Cells were treated for 1hour with vitamin C, and then washed twice in PBS, and fresh media was added and the cells incubated for another 48 hours. Their metabolic activity was measured using the MTT assay. The average and SEM of two separate experiments is shown.

Vitamin C and Catalase

To find out if the toxic effect of vitamin C was due to the production of hydrogen peroxide, the same experiment was repeated with catalase added to breakdown any hydrogen peroxide. However, catalase was not able to protect the cells indicating that vitamin C was not acting through hydrogen peroxide.

Cell cycle in TSC2 -/- cells

As a side note, in our experiments with vitamin C we also looked at the effect of high concentration vitamin C on cell cycle. The method used to investigate cell cycle measures the amount of DNA within the cells. While these experiments were inconclusive, we

noticed an unusual pattern with high passage TSC2 -/- cells that might be due to DNA instability in the cells. This pattern was not observed in lower passage TSC2 -/- cells, so it is tempting to speculate that the DNA in these cells becomes unstable over time. Further experiments would be needed to explore this theory further.

Hypoxia

There are conflicting reports about the effect of hypoxia (low oxygen) on TSC2 -/- cells [17,18]. We conducted preliminary experiments to see if the TSC2 -/- cells had a growth advantage under hypoxic conditions (oxygen concentration < 3%). The findings from these experiments did not indicate any differences in growth between the TSC2 +/+ and the TSC2 -/- cells.

Summary

Key findings with primary LAM and non LAM cells

- LAM and non LAM cells were not able to form spheres typical of cancer stem cells in stem cell media.
- LAM and non LAM cells upregulated stem cell genes in stem cell media.
- Primary LAM cells could not be distinguished from control non LAM smooth muscle cells by HMB45 staining, gp100 gene expression, estrogen receptor expression, response to estradiol or to the mTORC1 inhibitor, rapamycin.

Key findings with TSC2 -/- cells

- TSC2 -/- cells were more sensitive to rapamycin than TSC2 +/+ cells.
- Metformin had the same effect on TSC2 +/+ and TSC2 -/- cells.
- Rapamycin plus metformin was only slightly more effective than rapamycin on its own.
- TSC2 -/- cells survived better under low serum conditions.
- TSC2 -/- cells exhibited resistance to hydrogen peroxide treatment.
- High concentration vitamin C had a similar effect on TSC2 +/+ and TSC2 -/- cells.

Overall conclusion and thoughts

Taken together these results highlight the difficulty of using primary LAM cells in *in vitro* experiments as a model of LAM and suggest that changes might have occurred to the LAM cells in routine cell culture. On the other hand, our work with TSC2-/- cells reveals the potential for these cells to be more resistant than TSC2+/+ cells under certain conditions of stress. It is tempting to speculate that the stress resistance of TSC2-/- cells *in vitro*, even though only slightly above that of TSC2 +/+ cells, relates to the slowly progressing nature of LAM. However, it would be difficult to test this hypothesis and other avenues of research with the potential for improved treatment might be more fruitful. These should include trying to understand what makes LAM cells destructive in the lung, and learning how to prevent this destructive capacity.

References

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