Chapter 7: General Discussion

This thesis has focussed on the functional characterisation of a genetic subset of NSCLC characterised by loss of the tumour suppressor *LKB1* and activation of the oncogene *KRAS*. NSCLC has poor survival rates (~7% at 5years) (Coleman, et al. 2004). There is therefore a need for early detection and more targeted therapies. Identification of genetic subsets and full characterisation of consequences of the driver mutations should enable targeting of therapies to groups of patients defined by genetics. Through a combination of targeted approaches, expression analysis and NMR spectroscopy this study has shown that *LKB1* inactivation/*KRAS* activation are the key driver mutations in these cancers.

I identified a statistically significant association of *LKB1* inactivating and *KRAS* activating mutations in a subset of NSCLC cell lines (Mahoney, et al. 2009). Due to the crosstalk between the LKB1/AMPK/mTOR and RAS-MAPK pathways I hypothesised that this genetic subset may be more sensitive to targeted inhibition of MEK and mTOR. This genetic subset was more sensitive to MEK and mTOR inhibition compared to NSCLC with either *LKB1* or *KRAS* mutations alone or NSCLC wild type for both genes (Mahoney, et al. 2009). However the effects of MEK inhibition were not due to downstream effects on cyclin D1 as has been shown in melanoma (Solit, et al. 2006) but were possibly due to downstream effects on p70S6K. Dual inhibition was surprisingly neither additive nor synergistic which may be explained by the potent inhibition of phosphorylation of p70S6K by rapamycin, precluding any additional effect of CI-1040, confirming likely redundancy in the pathways specifically in this subset of NSCLC. The data suggests that *LKB1* loss and *KRAS* mutations appear to be the key driver mutations in this subset of NSCLC and newer generation MEK and/or mTOR inhibitors may represent therapeutic

options in the subset of patients with *LKB1/KRAS* mutant tumours. The work presented also suggested that the smaller subset of NSCLC characterised by *LKB1* loss and *BRAF* (*non-V600)* mutations which are insensitive to MEK inhibition but sensitive to mTOR inhibition. There are only a small number of cell lines with this combination of mutations and given the unclear role of *non-V600 BRAF* mutations in NSCLC, Therefore larger studies are needed to more definitely address this potentially important issue.

We further investigated the combination of *LKB1* loss and *KRAS* activation in NSCLC by examining the expression profile of this subset of NSCLC compared to non-*LKB1/KRAS* mutant NSCLC. The *LKB1/KRAS* mutant cell lines showed a distinct expression pattern characterised by alteration to the expression of a large number of metabolic genes. One finding of particular significance was the 18-fold higher expression of PDK4 and almost 6-fold lower expression of PDP1 in *LKB1/KRAS* mutant cell lines versus other NSCLC cell lines. PDK4 and PDP1 control the activation state of the pyruvate dehydrogenase complex (PDC) by phosphorylation. PDK4 phosphorylates and inactivates the complex, PDP1 dephosphorylates and activates it. The role of the PDC is to regulate entry of pyruvate into the TCA cycle. It has been hypothesised that PDC inactivation is a mechanism to create the Warburg Effect (Roche & Hiromasa 2007). The Warburg Effect is the switch to aerobic glycolysis, whereby cancer cells produce energy via lactate production rather than oxidative phosphorylation (Warburg, 1956). It was first observed in the 1950's but only recently are the mechanisms behind it being uncovered. I hypothesised that the altered expression of PDK4 and PDP1 would lead to inactivation of the PDC and production of increased amounts of lactate. The observation that the PDC could be being inactivated through altered expression of PDK4 and PDP1 apparently driven by presence of *LKB1* and *KRAS* mutations suggested a novel mechanism for creating the Warburg Effect. We further investigated this using NMR spectroscopy with

labelled glucose to identify significantly different metabolites between *LKB1/KRAS* mutant cell lines and control cell lines. The data presented in this thesis suggests that these cells produce significantly larger amounts of lactate. In addition they produce significantly more succinate which further indicates that the PDC is inactive. The PDC converts pyruvate to acetyl-coA through a series of enzymatic reactions, PDC inactivation would restrict entry of acetyl-coA into the TCA cycle resulting in a block on the conversion of oxaloacetate to citrate, leading to the build up of succinate. The build up of succinate has also been linked to pseudohypoxia through the inactivation of prolyl hydroxylases and consequent HIFα activation and stabilisation (Koivunen, et al. 2007; Selak, et al. 2005). This would likely give the cells a survival advantage in the demanding *in vivo* conditions.

The NMR data also revealed that this genetic subset would appear to rely heavily on both glucose and glutamine metabolism and indeed the expression data showed increased expression of glutamate metabolism genes such as glutaminase which is responsible for the conversion of glutamine to glutamate. Glutamine metabolism is an alternative to glucose metabolism and the resulting glutamate is converted to α-ketoglutarate to replenish TCA cycle intermediates (DeBerardinis, et al. 2007). Accelerated glutamine metabolism is associated with production of increased amounts of reducing equivalents which can be used in detoxification processes such as glutathione metabolism to deal with oxidative stress from reactive oxygen species (ROS). The expression data also showed altered expression in glutathione metabolic genes suggesting an overall increased capacity to deal with reactive oxygen species under conditions of oxidative stress. Overall the NMR spectroscopy data revealed this genetic subset differed greatly from the other NSCLC cell lines tested at the metabolic level. Interestingly the NMR spectroscopy data could be used to create a metabolic profile-based model of *LKB1/KRAS* mutant cancers that could be used to classify cancers of unknown genetic status into an *LKB1/KRAS* mutant “like”

category. The finding in this study that alterations at the transcriptional level to PDK4 and PDP1 could be the cause of the Warburg Effect has important therapeutic implications. Tumours have been shown to express the embryonic specific isoform of pyruvate kinase (PKM2) (Mazurek, et al. 2005; Dombrauckas, et al. 2005). Replacement of PKM2 with the PKM1 adult isoform reverses the Warburg phenotype and decreases the tumourgenicity of H1299 cells in nude mouse xenografts (Christofk, et al. 2008). Further work is needed to explore whether PDK4 inactivation or PDC reactivation are possible therapeutic targets in this subset of NSCLC to reverse the Warburg Effect. It would also be interesting to study whether *in vivo* these *LKB1/KRAS* mutant lung cancers have the same metabolic phenotype. The additional finding that these tumours rely heavily on both glutamine and glucose metabolism may suggest additional therapeutic opportunities such as inhibition of glutaminase. Whilst these may not be ideal targets in patients, targeting them *in vitro* may help identify more suitable *in vivo* targets.

The data presented here was obtained from cell lines which are grown in ideal nutrient and growth conditions. Even under ideal growth conditions the cells have an accelerated metabolic phenotype, suggesting that *in vivo* they would have a survival advantage. Further the *in vitro* phenotypic data presented make a strong case for these changes being "hard-wired" by the mutation states. Further investigation of the metabolic profile using other labelled metabolites such as glutamine would enable greater depth of knowledge into the metabolic profile and may provide further insights into potential therapeutic avenues. It has recently become apparent that *BRAF(V600)* or *RAS* activated melanomas, LKB1 is phosphorylated and AMPK signalling inactivated (Esteve-Puig, et al. 2009; Zheng, et al. 2009). It would be interesting to study whether the metabolic phenotype is recapitulated in different tumour types which appear to have *RAS-MAPK* activation and LKB1 protein inactivation through signalling mechanisms. Initial work examining

the expression profiles of these tumours should give an indication as to whether they are similar at the transcriptional level to the *LKB1/KRAS* mutant NSCLC discussed here.

We next investigated whether the sensitivity to CI-1040 and rapamycin was due to alteration to the expression signature described above. CI-1040 and rapamycin treatment had very little effect on the expression profile suggesting that the mechanism of compound sensitivity is separate to the expression profile/metabolic phenotype. A small number of genes in *LKB1/KRAS* mutant cell lines did show alterations in expression, including glutaminase which showed decreased expression upon CI-1040 and rapamycin treatment. Glutaminase was one of only three genes which showed alteration to expression after both treatments and given the metabolic data described above may warrant further investigation. In particular it might be informative to carry out similar NMR experiments in CI-1040/rapamycin treated NSCLC cell lines to more clearly address the role of glutamate in *LKB1/KRAS* mutant metabolic phenotype.

Due to the complexities in the signalling pathways and the many inputs mTOR receives we sought to directly inhibit the LKB1/AMPK/mTOR pathway upstream of mTOR to determine the contribution to the expression phenotype. Treatment of cell lines with the AMPK inhibitor, Compound C, revealed it had a paradoxically greater inhibitory effect on the proliferation rate of *LKB1/KRAS* mutant cell lines than other cell lines tested. This was not the case for an *LKB1null/KRAS* wild type cell line indicating that the effect was specific to the *LKB1/KRAS* mutant subtype. There are a number of possible explanations for this provocative finding. It is possible that despite *LKB1* inactivation there could be residual AMPK activity necessary for cell growth. For example other kinases such as TAK1 and CaMKK phosphorylate AMPK and some residual phosphorylation is observed in *LKB1* null cell lines (Carretero, et

al. 2007). If such is the case, then inhibition of AMPK in *LKB1/KRAS* mutant cancers may be an area that warrants further investigation as a potential therapeutic target. An alternative explanation is that the inhibitor has "off-target" effects that are reasonably specific to *LKB1/KRAS* mutant cancers. Compound C is also known as dorsomorphin and is a known inhibitor of BMP signalling (Hao, et al. 2008). Expression analysis of AMPK inhibitor treated cells did not yield any obvious explanations and showed no alteration to characterised AMPK targets such as fatty acid synthase (Zhou, et al.2001). GO term enrichment analysis did not reveal any enrichment for genes involved in BMP signalling but several known BMP targets such as ID1, 2 and 3 (Darby, et al. 2008; Lorda-Diez, et al. 2008; Shepherd, et al. 2008) showed decreased expression upon Compound C treatment. This alteration in expression however was not restricted to *LKB1/KRAS* mutant cell lines. The mechanism of growth inhibition upon Compound C treatment did involve apoptosis at lower concentrations in *LKB1/KRAS* mutant cell lines than control cell lines. Although the GO term enrichment analysis of AMPK inhibited cell lines did not reach statistical significance, the categories enriched were related to apoptosis. Thus, it would appear that the mechanism of growth inhibition from AMPK inhibition is different to that observed from MEK/mTOR inhibition as it involves an apoptotic response rather than a cytostatic response. In addition, contrary to expectation based on current signalling models PDK4 has higher expression in *LKB1/KRAS* mutant NSCLC cell lines than in control cell lines. The expression of PDK4 is normally promoted by AMPK and fatty acids, as observed in skeletal muscle (Houten, et al. 2009) therefore expression would be expected to be decreased in *LKB1null* cell lines. Further work is required to determine the mechanism of the inhibitor and whether it has any therapeutic potential, bearing in mind the role of AMPK in cellular energy homeostasis and whole-organism energy homeostasis.

The expression data suggested that *LKB1/KRAS* mutant cell lines had 70-fold higher expression of an aldo-keto reductase AKR1B10. Overexpression was further confirmed at the protein level. AKR1B10 is an aldo-keto reductase, an NADPH linked oxidoreductase, expressed normally in the small intestine. It metabolises aldehydes and ketones to their corresponding primary and secondary alcohols. AKR1B10 has been implicated in a number of cellular processes, two of which were investigated here. Penning et al. 2007 suggested that AKR1B10 plays a role in cancer through deregulation of retinoic acid signalling. We did not find any evidence for this in AKR1B10 expressing NSCLC cell lines. In addition Yan et al. 2007 reported that siRNA inhibition of AKR1B10 in colorectal cancer cells lead to a decrease in cell proliferation. When siRNA knockdown of AKR1B10 in NSCLC cell lines was carried out as part of the work presented here we did not see any effect on the proliferation rate of the cells. Thus role for AKR1B10 in NSCLC is still not clear. It should be noted that complete knockdown of AKR1B10 at the protein level was not achieved, therefore the interpretation of these data are tentative as there could still be sufficient residual activity to mitigate any knockdown effects. AKR1B10 has been suggested to play roles in chemoresistance and fatty acid metabolism through interactions with ACC and recent data has suggested AKR1B10 expression in the intestine is physiologically important for protecting against dietary and lipid-derived cytotoxic carbonyls (Zhong, et al. 2009). It may therefore play a more important role in metabolism of cytotoxic/chemotherapeutic compounds in NSCLC.

While work presented here has identified a new genetic subset of NSCLC, there are still a number of questions remaining. One such question is the link between *LKB1/KRAS* mutations and the expression signature. Further work on this subset of cancers should perhaps include investigation as to the role of MYC, as both RAS and LKB1 regulate the stability of the MYC protein. MYC is a well known downstream target of ERK1/2 (Sears, et al. 1999) and phosphorylation of serine-62 in the N-

terminus of MYC by ERK1/2 stabilises the MYC protein (Sears, et al. 2000). It has recently been reported that LKB1 may regulate the proteasomal degradation of the MYC protein (Liang, et al. 2009). Given the role of MYC in regulating metabolism, particularly in cancer (reviewed by Gordan, et al. 2007), it may provide the missing link between the alterations at the signalling level and how they result in the expression signature dominated by metabolic genes. Examination of the gene expression data suggests that MYC does not have increased expression specifically in this subset of NSCLC. In addition, *in vivo* work is now of particular importance, to determine which findings are relevant *in vivo* and how they can be exploited therapeutically.

With the advent of high-throughput drug screening and the knowledge of cancer genomes becoming more complete, research is beginning to deliver on the promise of "personalised medicine". Projects which combine complete genomic information of cell lines with therapeutic response will gradually categorise cancer into subsets defined by genetics and therapeutic response. This is of particular importance in lung cancer; with poor survival rates and a lack of early detection methods and an urgent need for more targeted therapies and biomarkers. Starting with the identification of genetic subset of lung cancer this project has yielded insights into the functional consequences of the mutations. Through targeted approaches, expression analysis and metabolic profiling it has provided greater understanding of a genetic subset of lung cancer. Although the picture is not complete the work presented here points to a number of promising avenues for further study which will hopefully contribute to the development of new molecular targets for therapy in NSCLC.