Chapter 6:

Functional analysis of AKR1B10 expression in *LKB1/KRAS* mutant NSCLC.

6.1 Introduction

As discussed in Chapter 4 we identified 70-fold higher steady state expression of AKR1B10 in *LKB1/KRAS* mutant cell lines compared to the control cell lines. AKR1B10 is not normally expressed in lung tissue but its expression has been found in approximately 30% of NSCLC, this led to the suggestion it may be a biomarker for NSCLC (Fukumoto, et al. 2005; Kim, et al. 2007).

Aldo-keto reductases are a superfamily of NADPH linked oxidoreductases which reduce aldehydes and ketones to their corresponding primary and secondary alcohols (Jez, et al. 1997a, b & 2001). They functionalise carbonyl groups by forming alcohols for conjugation reactions and release $NADP^+$ (Aksonas, et al. 1991; Grimshaw, et al. 1995). They are monomeric proteins of between 34-37kDa and exist across all phyla with 151 members in fifteen families. In humans there are thirteen members which act on a number of substrates including reactive lipid aldehydes, sugar aldehydes, steroids and ketoprostaglandins (reviewed by Jin & Penning 2007). As well they are implicated in xenobiotic metabolism as they metabolise various drugs and chemical carcinogens (Barski, et al. 2008). The three families in humans are AKR1, 6 and 7.

AKR1B10 belongs to the AKR1 family of aldo-keto reductases and is also known as small intestine like aldose reductase due to its limited expression, mainly in the small

intestine (Cao, et al. 1998). AKR1B10 has been found to overexpressed in a small number of cancers, in particular lung cancer with almost 30% of adenocarcinomas showing expression (Fukumoto, et al. 2005; Kim, et al. 2007). Following this it has been postulated to be a biomarker for NSCLC.

The role of AKR1B10 in cancer and indeed normal cells still remains unclear. It has been suggested that it is involved in vitamin A metabolism and retinoic acid signalling due to its high affinity for retinol, with a K_{cat}/K_m 100-fold higher than AKR1B1 and other family members (Crosas, et al. 2003; Gallego, et al. 2006; Gallego, et al. 2007; Ruiz, et al. 2009). Retinoic acid (RA), the oxidised form of vitamin A (retinol) is essential for a broad range of biological processes including development differentiation and growth (Lotan, 1981). RA exerts its functions by binding to nuclear receptors (Heyman, et al. 1992). It has been suggested that in cancer AKR1B10 metabolises retinal back to retinol and prevents the formation of retinoic acid, the ligand for RXR (Penning, et al. 2007) (Figure 6-1). However evidence for this has yet to be shown. There are natural retinoids which include 9-*cis* retinoic acid (9-*cis*-RA) and *trans*-retinoic acid (*trans*-RA) which can function as ligand inducible transcription factors of retinoic acid receptors 9-*cis*-RA can interact with both retinoic acid receptors (RARα and β) and retinoid X receptor (RXR) whereas *trans*-RA can only interact with RAR (Zhang, et al. 1993). There are 3 isoforms of each receptor α, β and γ, which show different expression patterns through development and differentiation. The receptors heterodimerise to modulate gene expression through recognition of retinoic acid response elements (RAREs) (Zhang, et al. 1993). They also autoregulate their expression (Hoffmann, et al. 1990).

Retinoids have been identified as potential anti-cancer agents due to their apoptotic and differentiation effects (Lotan, et al. 1991; Hofmann, 1992), however results thus far have been mixed. One major trial, the CARET (The Carotene and Retinol

Efficacy Trial) trial was halted early (Omenn, et al. 1996). The CARET trial began in 1985 and stopped in 1996, it was a randomized, double-blind, placebo-controlled trial of the cancer prevention efficacy and safety of a daily combination of beta-carotene and retinyl palmitate in almost twenty thousand persons at high risk for lung cancer. It was found that not only was there no evidence for benefit in lung cancer, there was evidence that it had had a harmful effect on lung cancer incidence and mortality (Omenn, et al. 1996). Given the possible role of AKR1B10 in retinoic acid metabolism, one could hypothesise that expression of AKR1B10 in persons with lung cancer would lead to an exacerbation of their condition upon treatment with beta-carotene. Other studies have implicated AKR1B10 in proliferation and DNA synthesis (Yan, et al. 2007), metabolism of daunorubicin and oracin (Martin, et al. 2006) and fatty acid synthesis, through the stabilisation of acetyl-coA carboxylase in breast cancer cells (Ma, et al. 2007).

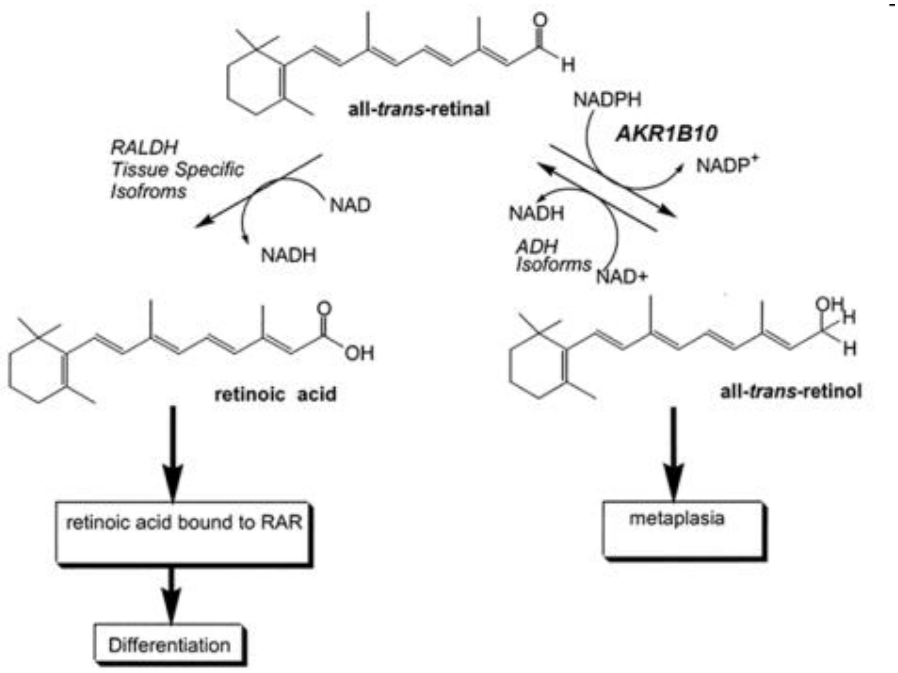

Figure 6-1. The hypothesised role of AKR1B10 in retinoic acid signalling (Figure from Penning et al. 2007). AKR1B10 has been shown to have the highest affinity of all aldo-keto reductases for retinal. Retinoic acid, the oxidised form of vitamin A is essential for differentiation and a number of cellular functions and exerts its functions by binding to nuclear receptors and modulating gene expression. It has been suggested that in cancer it metabolises retinal back to retinol and prevents the formation of retinoic acid, the ligand for RXR.

In this Chapter I examine whether AKR1B10 is an important functional determinant of the *LKB1/KRAS* mutant phenotype by investigating the possible role of AKR1B10 in retinoic acid signalling and cellular proliferation in NSCLC cell lines.

6.2 Results

6.2.1 AKR1B10 protein expression in *LKB1/KRAS* mutant NSCLC cell lines

To determine whether AKR1B10 mRNA expression resulted in over-expression of the protein in *LKB1/KRAS* mutant NCSLC cell lines, immunoblot analysis of AKR1B10 was carried out on a panel of NSCLC cell line protein lysates. This antibody recognises a single band at approximately 36kDa (Figure 6-2).

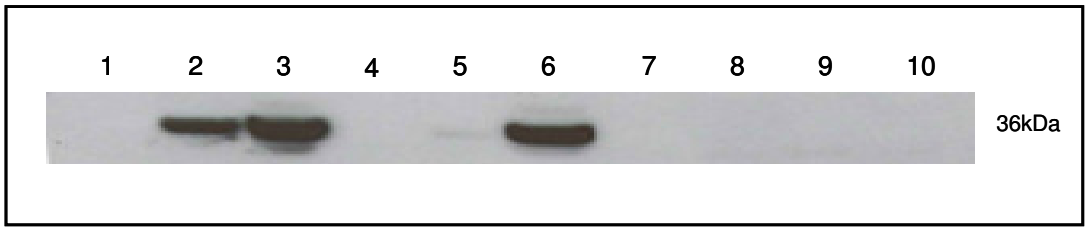

Figure 6-2. AKR1B10 is expressed in *LKB1/KRAS* NSCLC cell lines. Immunoblot analysis of AKR1B10 expression in 10 NSCLC. 1 – CAL-12T (*LKB1/BRAFmut*), 2 - NCI-H460 (*LKB1/KRASmut*), 3 – A549 (*LKB1/KRASmut*), 4 – NCI-H1437 (*LKB1null*), 5 – NCI-H1563 (*LKB1null*), 6 – NCI-H1734 (*LKB1/KRASmut*), 7 – NCI-H1838 (*WT*), 8 – NCI-H1975 (*WT*), 9 – NCI-H1993 (*LKB1null),* 10 – NCI-H2009 (*KRASmut*).

As figure 6-2 shows expression of AKR1B10 protein is restricted to *LKB1/KRAS* mutant NSCLC cell lines, confirming the result from the expression data. There is no expression of AKR1B10 in the *LKB1BRAF* mutant cell line (CAL-12T) consistent with this being a unique genetic subset of NSCLC

6.2.2 siRNA knockdown of AKR1B10 does not affect proliferation rate in AKR1B10 expressing NSCLC cell lines

To determine the role of AKR1B10 on proliferation we used siRNA to knockdown AKR1B10 expressio. Previous work by Yan, et al. 2007 in colorectal cancer cell lines showed significant effects of AKR1B10 knockdown on cell proliferation, 7 days after knockdown. It was therefore decided to assess proliferation rate in the NSCLC cell lines after 7 days of AKR1B10 knockdown. To estimate the level of knockdown being achieved 96hrs after transfection, cells were seeded to 6-well plates and double transfected with four single siRNAs (S1, S2, S3 and S4) to AKR1B10, 24 and 72hrs after cell seeding and the following day cells harvested and protein extracted using RIPA buffer (Figure 6-3).

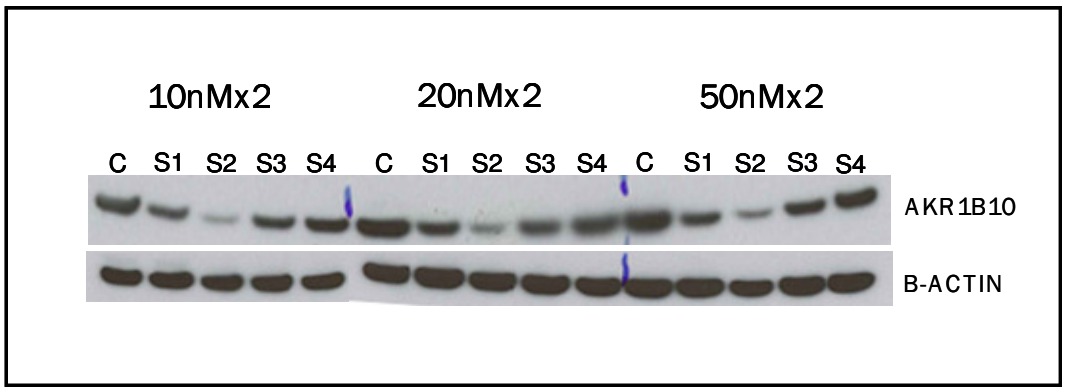

Figure 6-3 Immunoblot of siRNA knockdown of AKR1B10 in NCI-H460 cells. Cells were treated with; 2x10nM siRNA, 2x20nM siRNA or 2x50nM siRNA to AKR1B10 24 and 72hrs after cell seeding, protein was then harvested the day after the final treatment. C – Control not transfected with siRNA, S1-4 - single siRNAs to AKR1B10.

S2 was the only siRNA to have a significant effect on the AKR1B10 protein levels, with knockdown estimated to be 80% using ImageJ software. Increasing the amount

of siRNA from 10nm to 50nm did not increase the level of knockdown. This is despite a <95% transfection efficiency in all cell lines used (data not shown).

To estimate the proliferation rate after AKR1B10 knockdown using siRNA S2, cells were seeded to 96-well plates in quadruplicate and double transfected as described above with 10nM siRNA to AKR1B10 and proliferation rate assessed 7days after knockdown. Controls to estimate the level of toxicity were non-targeting siRNA, lipofectamine™ 2000 control and a media untreated control. Proliferation rate was estimated using CyQuant® reagent 7 days after the first transfection, averages taken from the quadruplicates and relative proliferation rate estimated by comparison to non-targeting siRNA treated cells (Figure 6-4). Five cell lines were used, three expressing AKR1B10 (A549, NCI-H460 and NCI-H2030) and two without AKR1B10 expression (NCI-H1792, NCI-H1838). Figure 6-4 shows there was no significant effect on the proliferation rate of cell lines transfected with 10nM siRNA to AKR1B10 7 days after transfection, suggesting that AKR1B10 may not play a role in NSCLC cell proliferation. Alternatively it could be that the residual 20% expression of AKR1B10 is sufficient for normal growth in these cell lines.

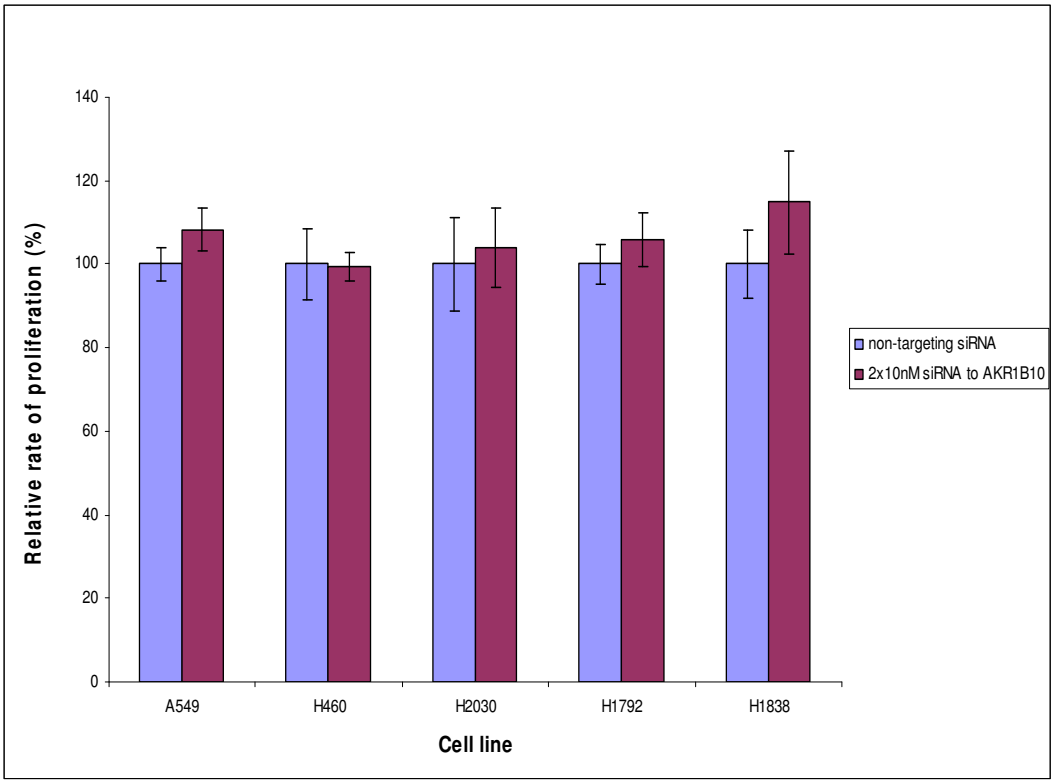

Figure 6-4 Relative proliferation rate of NSCLC cell lines 7 days after transfection with 10nM siRNA to AKR1B10. Values shown here are ± stdev, n=8 from 2 independent experiments. There are no significant differences in proliferation rate of cells treated with non-targeting siRNA (blue bars) and cells treated with 10nm siRNA to AKR1B10 (purple bars). AKR1B10 positive cell lines (A549, NCI-H460 and NCI-H2030), AKR1B10 negative cell lines (NCI-H1792 and NCI-H1838).

6.2.3 AKR1B10 does not play a role in retinoic acid signalling in NSCLC cell lines

AKR1B10 has been found to have high affinity for the retinal family of metabolites (Crosas, et al. 2003; Gallego, et al. 2006; Gallego, et al. 2007; Ruiz, et al. 2009) and is therefore thought to play a role in retinoic acid signalling by preventing the formation of retinoic acid which would normally bind to nuclear receptors. Therefore we hypothesised that AKR1B10 expression in cells would normally prevent the formation of retinoic acid, thus leading to a block on differentiation and increase cell proliferative potential. Treating *LKB1/KRAS* mutant cell lines with retinoic acid derivatives (9-*cis* and ATRA) would therefore be expected to cause a decrease in proliferation rate in cells expressing AKR1B10 (*LKB1/KRAS* mutant cell lines), whereas addition of beta-carotene would have little effect (Figure 6-5). Six cell lines, three with AKR1B10 expression (NCI-H460, A549, NCI-H2030) and 3 without (NCI-H1838, NCI-H1792, NCI-H2009) were treated with beta-carotene (0-40μM, all *trans*-retinoic acid (ATRA) and 9 *cis*-retinoic acid (9 *cis*-RA) (0-20μM) for 72hrs. After 72hrs cells were assayed for proliferation rate using CyQuant reagent.

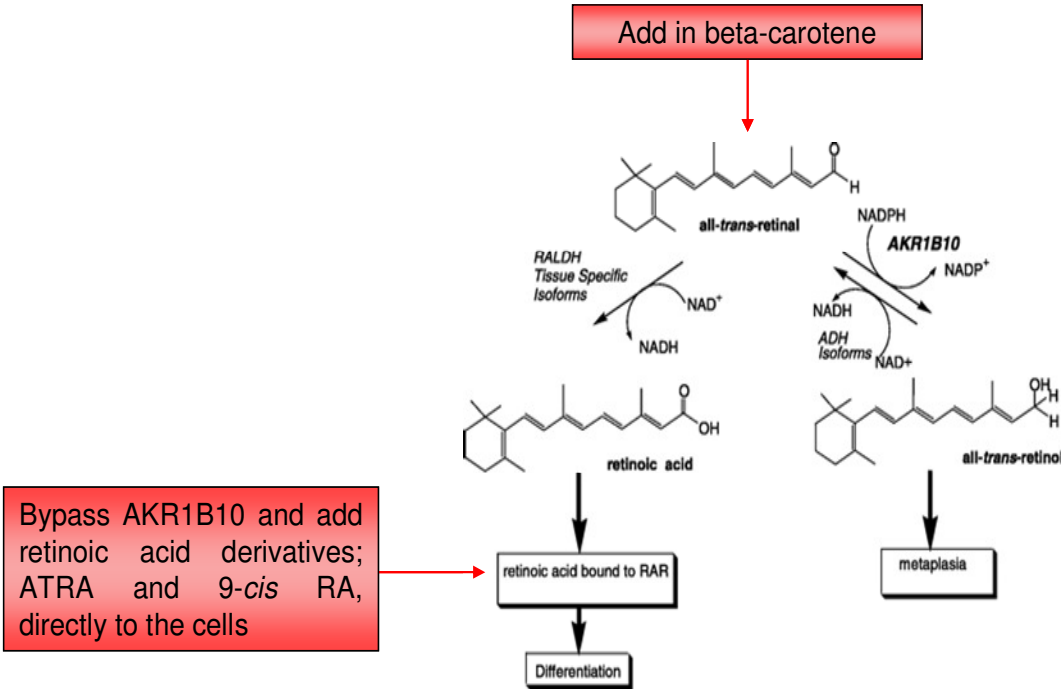

Figure 6-5 Experimental plan to examine whether AKR1B10 plays a role in retinoic acid signalling in NSCLC cell lines. It was hypothesised that addition of retinoic acid derivatives to the cells would bypass AKR1B10 metabolism and induce growth inhibition specifically in *LKB1/KRAS* mutant cell lines, whereas addition of beta-carotene should have little effect on cell proliferation.

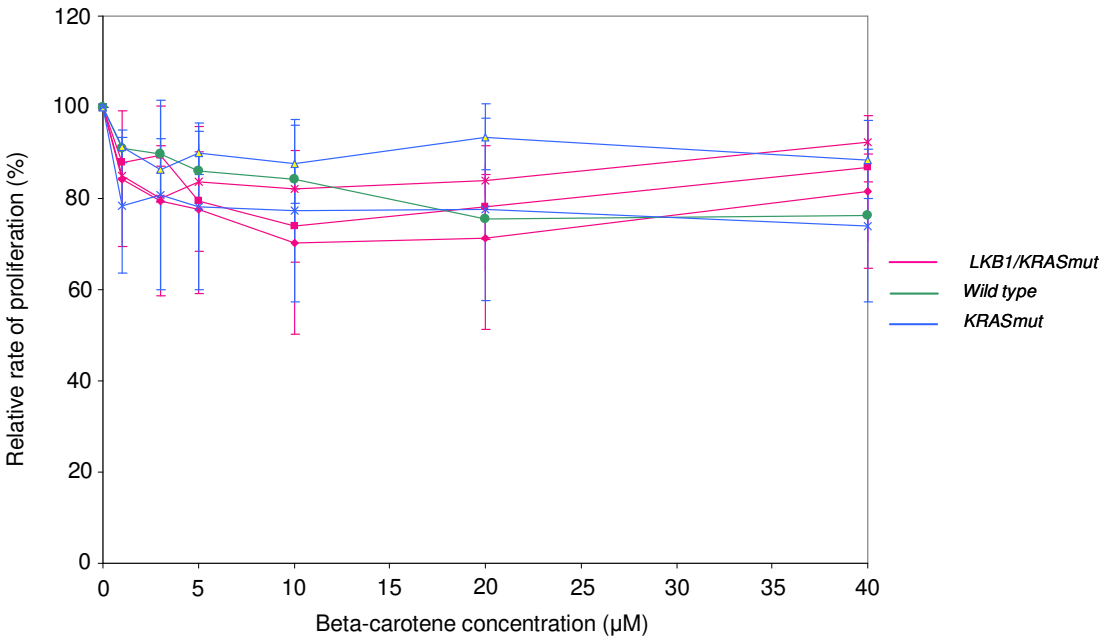

Figure 6-6 Treatment with beta-carotene has no significant effect on proliferation rate of NSCLC cell lines. NSCLC cell lines positive for AKR1B10 expression (NCI-H460, A549, NCI-H2030) or negative (NCI-H1792, NCI-H2009, NCI-H1838) were treated with beta-carotene (0-40μM) for 72hrs. Relative rate of proliferation was then calculated using CyQuant® dye and measured relative to the untreated control. Results shown here are from at least 2 independent experiments (n≥8) ± stdev.

Figure 6-6 shows the relative proliferation rate after 72hrs of beta-carotene treatment. This experiment was hampered by the insolubility of beta-carotene, which is well documented (Palozza, et al. 2006). However, the data suggests that addition of beta-carotene to the cells has no discernable effects on proliferation rate regardless of AKR1B10 expression.

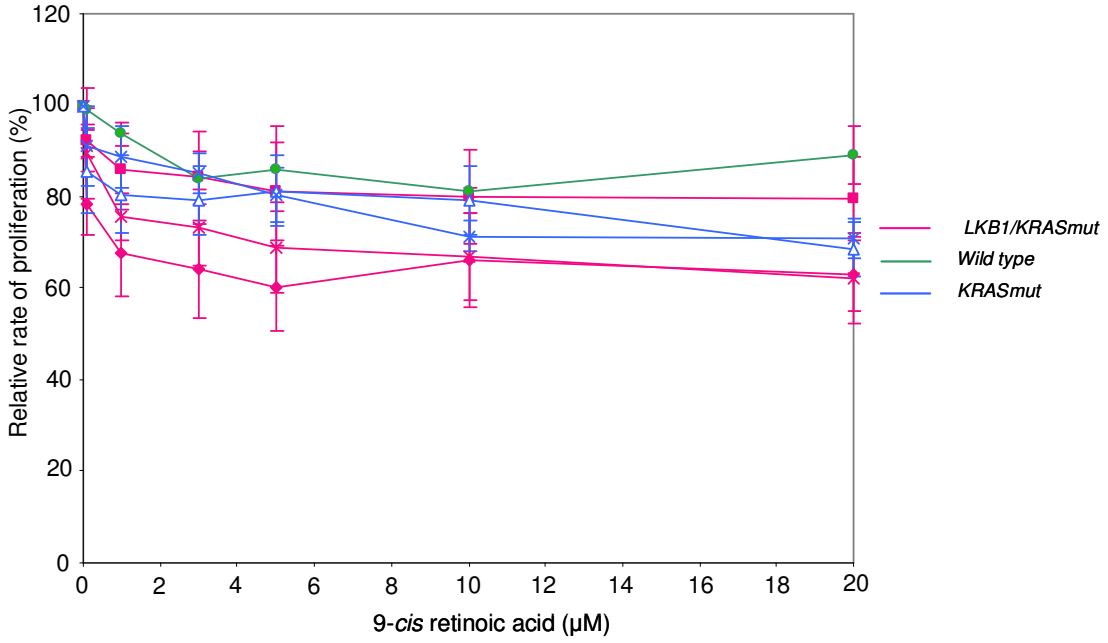

Figure 6-7 Treatment with 9-*cis*-retinoic acid has no significant effect on proliferation rate of NSCLC cell lines. NSCLC cell lines positive for AKR1B10 expression (NCI-H460, A549, NCI-H2030 (pink lines)) or negative (NCI-H1792, NCI-H2009, NCI-H1838 (blue and green lines)) were treated with 9-*cis*-retinoic acid (0-20μM) for 72hrs. Relative rate of proliferation was then calculated using CyQuant® dye and measured relative to the untreated control. Results shown here are from at least 2 independent experiments (n≥8) ± stdev.

Figure 6-7 shows the relative rate of proliferation rate of NSCLC cell lines after 72hrs of 9-*cis*-RA treatment, which might be expected to cause a decrease in proliferation rate in *LKB1/KRAS* mutant cell lines. There is no significant difference in the proliferation rate of NSCLC cell lines expressing AKR1B10 (*LKB1/KRASmutant*) upon treatment with 9-*cis*-RA which acts as a ligand for both RAR and RXR receptors. There is perhaps a trend for the *KRAS* mutant cell lines regardless of AKR1B10 expression to be more sensitive to 9-*cis* RA (blue and pink lines in Figure 6-7), however this is difficult to determine as there is only one cell line without a *KRAS* mutation (green line in Figure 6-7).

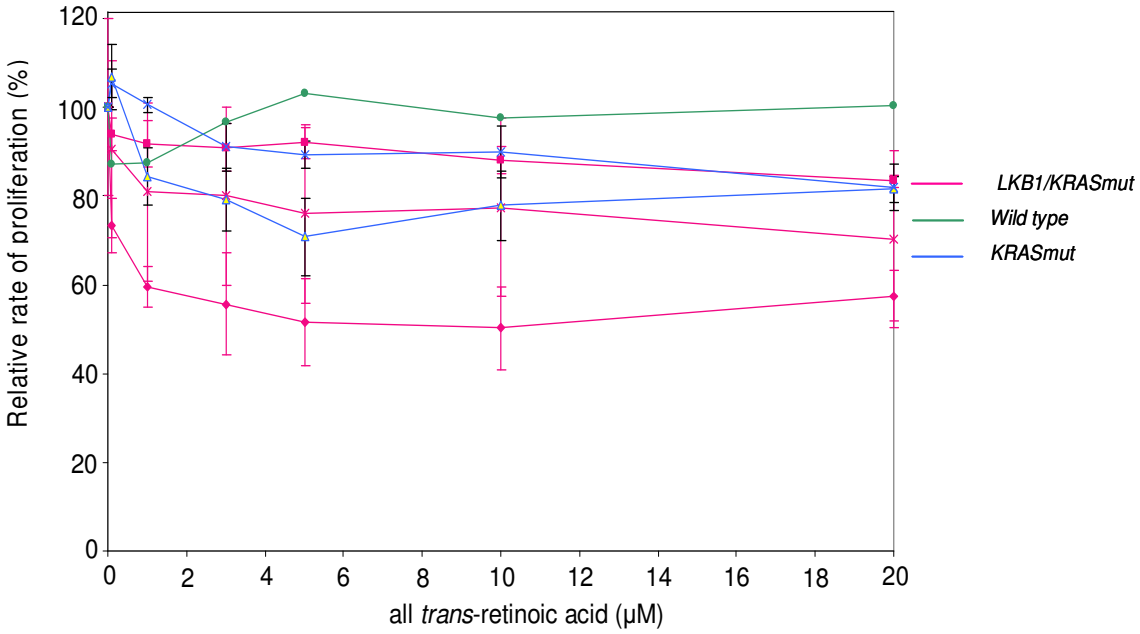

Figure 6-8 Treatment with All-*trans*-retinoic acid treatment (ATRA) has no significant effect on proliferation rate of NSCLC cell lines. NSCLC cell lines positive for AKR1B10 expression (NCI-H460, A549, NCI-H2030) or negative (NCI-H1792, NCI-H2009, NCI-H1838) were treated with ATRA (0-20µM) for 72hrs. Relative rate of proliferation was then calculated using CyQuant® dye and measured relative to the untreated control. Results shown here are from at least 2 independent experiments (n≥8) ± stdev.

Figure 6-8 shows the relative rate of proliferation rate of NSCLC cell lines after 72hrs of ATRA treatment, which might be expected to cause a decrease in proliferation rate in *LKB1/KRAS* mutant cell lines. Treatment with ATRA has no significant effect on the proliferation rate of NSCLC cell lines expressing AKR1B10 (*LKB1/KRASmutant*). Again, there is a trend for the *KRAS* mutant cell lines to be more sensitive to ATRA regardless of AKR1B10 expression and the effect is more pronounced with ATRA than 9-*cis* RA (blue and pink lines in Figure 6-7), however this is difficult to determine as again there is only one cell line without a *KRAS* mutation (green line in Figure 6-7).

6.3 Discussion

AKR1B10 is an aldo-keto reductase implicated in retinoic acid signalling, proliferation, fatty acid metabolism and xenobiotic metabolism (Penning et al. 2007; Yan, et al. 2007; Martin, et al. 2006; Ma, et al. 2007; Martin, et al. 2009). It has also been postulated to be a biomarker for NSCLC after expression was observed in approximately 30% of primary tumour samples (Fukumoto, et al. 2005; Kim, et al. 2007). We observed a correlation of AKR1B10 expression both at the mRNA level and at the protein level with *LKB1/KRAS* mutant NSCLC cell lines specifically.

We sought to determine the role AKR1B10 expression might be playing in the proliferation of NSCLC cell lines using siRNA knockdown. Double transfection of 10nM siRNA only gave 80% knockdown at the protein level and increasing the siRNA concentration did not increase the level of knockdown. In all cell lines tested there was no obvious effect of AKR1B10 knockdown on proliferation rate after 7 days, which suggests AKR1B10 does not play an important role in NSCLC proliferation. However, there was still approximately 20% expression of AKR1B10 protein and this could still be enough to carry out the functions of AKR1B10 in proliferation in NSCLC cell lines. Yan, et al. 2007 used two siRNAs to knockdown AKR1B10 expression in colorectal cancer cell lines, 60-95% knockdown at the protein level resulted in a 50% decrease in cell growth, indicating that in colorectal cancer cell lines AKR1B10 expression is important for cell proliferation.

AKR1B10 has also been proposed to play a role in retinoic acid signalling. Investigation of this in NSCLC cell lines expressing AKR1B10 has suggested that it does not. Treating cell lines with retinoic acid derivatives ATRA and 9-cis-RA did not have any significant effects on proliferation rate of cells expressing AKR1B10, nor did it affect the morphology of the cells (data not shown). There was a trend for *KRAS*

mutant cell lines to be sensitive to retinoic acid treatment regardless of AKR1B10 expression, however the data is not conclusive for this and a bigger panel of cell lines would need to examined.

Altogether these data suggest AKR1B10 does not play a role in the proliferation of NSCLC cell lines nor does it appear to play a role in retinoic acid signalling. Further work is required to determine the function of AKR1B10 expression in NSCLC and identify its substrate(s). Recent data has suggested a role in xenobiotic metabolism and metabolism of toxic lipid aldehydes which occur as a result of reactive oxygen species damaging cell membranes (Zhong, et al. 2009; Martin, et al. 2009).