University of Glasgow | College of Medical, Veterinary & Life Sciences

# Head of College Scholars List Scheme

## Summer Studentship

## Report Form

This report should be completed by the student with his/her project supervisor. It should summarise the work undertaken during the project and, once completed, should be sent by email to: jill.morrison@glasgow.ac.uk within four weeks of the end of the studentship.

1. Student

   Surname: Malik Forename: Natasha

   E-mail address: 1101426m@student.gla.ac.uk

2. Supervisor:

   Surname: Michie Forename: Dr. Alison M.

   E-mail address: Alison.Michie@glasgow.ac.uk

3. Research Project Report

   3.1 Project Title (maximum 20 words):

   Defining the molecular events that regulate PKCβII expression in a chronic lymphocytic leukaemia mouse model.

   3.2 Project Lay Summary (copied from application):

   Chronic lymphocytic leukaemia (CLL) is the most common blood cancer in the UK, and remains incurable. While a specific trigger for this cancer has not been defined, protein kinase CbetaII (PKCβII) is highly expressed in CLL cells and is linked with a poorer prognostic outcome. In this project, we will investigate mechanisms that regulate high expression of PKCβII in CLL cells. To achieve this, we will use a mouse model for CLL, in which we can track the stage at which PKCβII expression is upregulated, thus giving us the opportunity to elucidate the mechanisms that enable this event to occur.

3.3 Start Date: 1/7/2013 Finish Date: 10/8/2013

3.4 Original project aims and objectives (100 words max):

We hypothesize that deregulation of PKCβ expression may be linked to leukaemogenesis in CLL, and propose to investigate the mechanism(s) involved in this process. To achieve this we will utilise a mouse model of CLL leukaemogenesis, which during the course of leukaemia development specifically upregulates PKCβII expression. This is an excellent model system to delineate the molecular mechanisms that regulate *prkcb* promoter activity, at the level of:
1. transcription factor (TF) regulation (e.g. Sp1);
2. post-transcriptional level (e.g. ERK-MAPK signalling).
3. epigenetic regulation (e.g. methylation);
In this way we will delineate the molecular mechanisms that regulate *prkcb* transcription in our PKCα-KR-mediated CLL mouse model. Moreover, these studies may elucidate novel targets for the next generation of therapies to combat CLL.

3.5 Methodology: Summarise and include reference to training received in research methods etc.

(250 words max):

To obtain CLL cells, foetal liver (FL) from mice were retrovirally transduced with kinase inactive PKCα (PKCαKR) resulting in CLL like disease. This models several aspects of poor prognostic human CLL including elevated proliferation, upregulated expression of tyrosine kinase ZAP10, upregulation of PKCβII expression, and elevated ERK phosphorylation. Thus, to address *prkcb* regulation, the following experiments were set up:

1. PKCαKR cells were treated with 4 different concentrations of mithramycin (MM) (50 nM, 100 nM, 200 nM, 400 nM) in tissue culture. As an inhibitor of Sp protein family, MM treatment would determine if there was a difference in the expression levels of PKCβ mRNA. To enable this experiment, primary stem cells were differentiated into B cells, and then treated with MM, prior to preparing mRNA. mRNA was converted to cDNA using reverse transcriptase and gene expression was assessed by qRT-PCR.
2. PKCαKR cells were treated with MEK inhibitor AZD6244 (a therapeutic compound currently in clinical trials) at 100 nM and I μM to determine whether a link exists between elevated ERK signalling and upregulated PKCβII expression. PKCβII expression, together with expression of other genes including *blnk*, *bcl2*, *mcl1*, *tcl1, vegfα, lef1* were assessed by performing a qRT-PCR as discussed above. In addition, protein lysates were prepared from cells treated with AZD6244 for 24 and 48 hrs, then Bradford assays were carried out to assess protein concentration. Thereafter, protein expression of PKCβII and phospho-ERK were assessed by Western blotting.
3. PRKCB has shown to be hypometylated in CLL cells. Thus we induced hypomethylation in control (MIEV) cells by treating them with the methyltransferase inhibitor 5-Azacytidine and assessing PKCβ expression levels using the same methods.

Furthermore, troubleshooting approaches such as pre-amplification methods were also carried out to enhance the level of cDNA for qRT-PCR reactions.

3.6 Results: Summarise key findings (300 words max). Please include any relevant tables or images as an appendix to this report:

1. Control B cells (MIEV) and PKCαKR-expressing cells were used in all the experiments mentioned above. Thus to phenotypically characterise that the cells that are being used are indeed B cells, flow cytometry of MIEV and PKCαKR cells at day 7 (FL5), 14 (FL3) and 21 (FL1) was carried out and analysed. Figure 1a shows that PKCαKR cells are more GFP+ than the MIEV cells. GFP (Green Fluorescent Protein) is a fluorescent protein which just shows that the cells are expressing the retroviral vector. As PKCαKR cells proliferate more than MIEV cells, it is seen that PKCαKR cultures contain a higher percentage of GFP+ cells, which increases as the co-cultured progresses, due to out-competing GFP- cells. However in the MIEV co-culture a clear GFP- population is maintained throughout the culture period. The GFP+ cells are then isolated and labelled for CD19, which is a B cell marker (Figure 1b). More PKCαKR cells have converted into B cells as compared to the MIEV cells at day 7 of the co-culture. This is exacerbated with increasing number of days they are cultured. Analysis of CD23 and CD5 expression, both of which are CLL markers, in CD19+ GFP+ cells revealed that neither MIEV or PKCαKR cells are completely positive for both the markers. However, in the day 7 PKCαKR culture, some cells express CD5±CD23, which is not evident in the MIEV control cells (Figrue 1c). Western blot analysis shows the upregulation of PKCβII protein over time (D10, D17, D24) in PKCαKR cells (Figure 1d). Furthermore, this can be compared to the β-Tubulin control, which remains almost the same in the three samples.

2. PKCαKR cells were treated with Mithramycin and gene expression of PKCβII (*prkcb*) along with other genes including *bcl-2, vegf-alpha, mcl-2, blnk, lef-1* were assessed. These genes were assessed as they have been shown to be regulated by Sp1 in human CLL cells and therefore are being assessed in mice cells, which is seen in Figures 2a-d. All the figures are calibrated to the reference gene (GAPDH), which is why the untreated samples have no gene expression. Figure 2a shows that one biological replicate has the gene of interest, PKCβII up regulated (FL2) whereas in the FL3 mice, it is down regulated. Furthermore, 20% FCS (mutes the signals) was added in FL2 which expectedly showed little change in expression. Figure 2b-d show a clear down regulation in PKCβII gene expression in both FL1 and FL2 mice cells. *blnk* also appears to be downregulated with MM treatment. *mcl-1*, on the other hand, is upregulated regardless of MM. In addition, to enhance the signals of the cDNA in the qRT-PCR, the samples were pre-amplified (Figures 2c & 2d), both of which show a clear trend as discussed above. The differences in results in Figure 2a and Figures 2b-d will be discussed in the next section.

3. PKCαKR cells treated with AZD6244 (a MEK inhibitor) showed a downregulation of PKCβII gene expression when treated with 100nM AZD6244, but interestingly, almost no change when treated with a higher concentration, 1 μM AZD6244 (Figure 3a). Contrastingly, Figure 3b shows 2 biological replicates of mice foetal cells treated with AZD6244, and they show a upregulation in PKCβII gene expression. Expression of the other genes did not show a reliable pattern between the two graphs. This shows that AZD6244 does not seem to be having an effect on PKCβII at the mRNA level. Nevertheless, in Figure 3c, it is clearly seen that PKCβII protein expression decreases with increasing concentration of the drug added. A similar trend is seen for P-ERK (compared to the controls β-Tubulin and total-ERK respectively.) This shows that there is a clear inhibition of PKCβII expression and p-ERK activity at the protein level.

4. When MIEV cells (control) were treated with 5-Azacytidine, the drug to induce hypomethylation in the cells, the PKCβII gene expression reduced (Figure 4). This is interesting because human CLL cells over expressing PKCβII gene are hypomethylated. However, instead of an up regulation, a down regulation of the gene is observed. But, this experiment was performed just once and thus further study is needed to come to a certain finding.

3.7 Discussion (500 words max):

In CLL cells, there has been observed up regulation of PKCβII expression. Thus we tried to find different mechanisms to try and regulate this expression. We hypothesised that inducing a CLL like disease from haemopoetic stem cells in mice, obtaining PKCαKR cells and treating them with drugs and assessing them at the mRNA and protein levels at different levels would lead to substantial results.

To confirm the increasing proliferation of PKCαKR cells, flow cytometry analysis of MIEV and PKCαKR cells was done which showed that PKCαKR cells have a tendency to behave more similarly to stem cells at an early stage (Fig 1b and 1c). As D7 PKCαKR cells scatter around the graph, it indicates that they have the potential to develop into different cell lineages (CD11b+). Previously published work in the lab showed that a sub-population of PKCa-KR expressing cells do maintain multi-lineage potential, while the majority rapidly commit to the B cell lineage. These findings supported by the data shown here.

Western blots of untreated samples showed increasing levels of PKCβII expression (Fig 1d). By treating PKCαKR cells with mithramycin, which is an Sp1 inhibitor, we hypothesised that it would inhibit the protein binding at the promoter region thus leading to a downregulation of *prkcb.* One of the results was rather stochastic as in Figure 2a, one biological replicate (FL2) treated with 200nM MM showed a up regulation of PKCβII while another replicate (FL3) showed a down regulation of the protein. This could be due to the protein and how it functions in mice as opposed to humans. For instance, the Sp1 protein in also involved in the cell cycle, and thus further trials and investigation needs to be done in order to come up with a finding. However, the observations were mostly concurrent with the hypothesis (Fig. 2b-d). Certain samples needed to be amplified in order to enhance the signals at the DNA level, which enabled us to get more robust results.

To address the role of the ERK-MAPK signalling pathway in PKCbII expression PKCαKR cells were treated with AZD6244 (as this drug is a MEK inhibitor, which is the kinase upstream of ERK-MAPK), which would expectantly inhibit PKCβII expression. Results, as discussed, are rather inconsistent at the mRNA level. One reason for this could be due to the fact that MEK regulation is downstream of the PKCβII gene and thus the inhibitor might not have any affect on the gene expression at all. However, the treatment with the drug for 24 and 48 hrs clearly shows a down regulation of PKCβII expression at the protein level (Fig. 3c). This confirms that AZD6244 is inhibiting the ERK-MAPK pathway of PKCβII. Also, doing a densitometry would show the exact difference in protein expression with different concentrations and also with different times of treatments (24 and 48 hours). Thus the hypothesis seems to be consistent at the protein level.

The last part of the project involved treating the cells with 5-Azacytidine. As human CLL cells show an upregulation of PKCβII expression along with hypomethylation, MIEV cells and not PKCαKR cells were treated with the drug to induce hypomethylation in the cells. Interestingly, the results showed the complete opposite results as to what was expected. There was a down regulation of the gene of interest with increasing concentration of the drug as opposed to the opposite (Figure 4). This could be due to the viability of the cells. Also, it should be taken into consideration that this experiment was performed just once and thus further study is needed to come to a conclusion.

In conclusion, we have clearly demonstrated that PKCbII expression is regulated at the transcriptional level upon treatment with mithramycin, suggesting a potential role for the

transcription factor Sp1. Moreover, the ERK-MAPK pathway regulates PKCbII expression at the protein level. Further studies will elucidate the molecular mechanisms surrounding these findings.

4. Reflection by the student on the experience and value of the studentship (300 words max):

Having done 6 weeks of this project was very rewarding. Having had little experience in the laboratory and having to learn and practice different techniques was a challenge which was made easier with the help and support which I received from my colleagues in the Alison Michie Group. I was always encouraged to ask questions and all my queries were answered in the best possible manner as they could by giving examples of past papers and by giving me different sources to learn more from. Even if I was unclear about some of the concepts, they would go over it again with me till I was comfortable with what we were doing, and why, which was highly beneficial. The work ambience was healthy which further motivated me. I was never put down in any way and was just encouraged to perform better even if my experiments had a few errors.

Weekly meetings and presentations increased my confidence levels and encouraged me to present my data statistically and analyse my findings. Furthermore, I had to keep a record of all my data in a Lab book which was great way for recording the found data for analysis. After I had learned most of the techniques, I was free to come into the lab and perform my own experiments which included carrying out Bradford Assays, PCR reactions and qRT-PCR reactions at my own time and plan them accordingly which boosted my confidence and made me feel responsible for my experiments.

From a holistic view, I had a great experience. It was a pleasure to work and learn alongside Dr. Alison Michie and her group, and hopefully I get the opportunity to do so again.

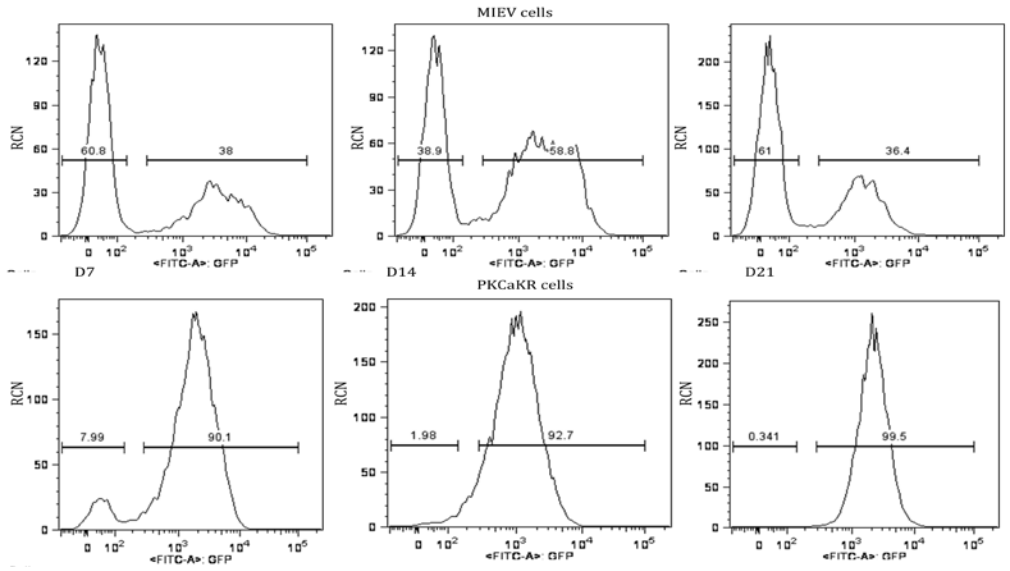
5. Dissemination: (note any presentations/publications submitted/planned from the work):

This project was based on a grant application that has been funded, but not started yet (start date 1st October). Therefore in the next couple of years we plan to build on this research and submit papers in the next 2 – 3 years.

6. Signatures: Supervisor Date Student Date

Appendix:

a)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60.8
38
D7
38.9
58.8
D14
61
36.4
D21
PKCaKR cells
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90.1
1.98
92.7
0.341
99.5
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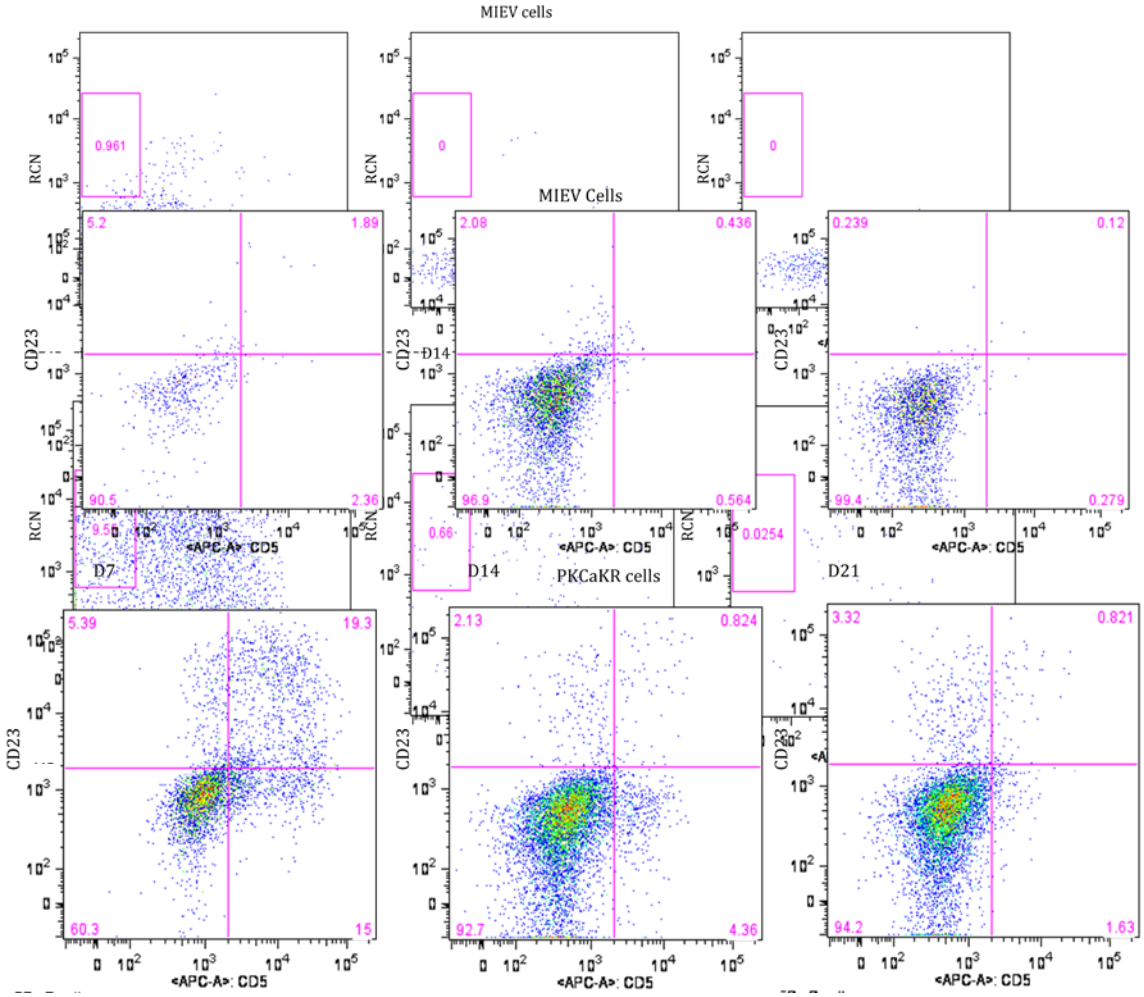
b)


MIEV cells
MIEV Cells
PKCaKR cells
RCN
CD23
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D14
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D21
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c)

d)

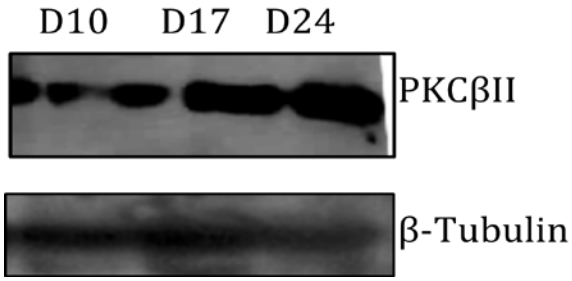


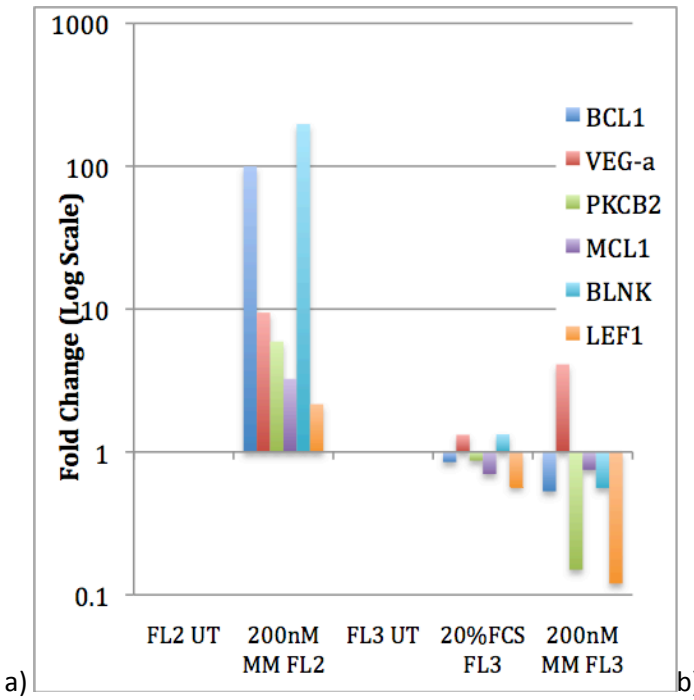

Figure 1: Phenotypic characterisation of control (MIEV) and PKCαKR cells during OP9 co-culture.

a) GFP+ expression on MIEV and PKCa-KR cells. The first row shows MIEV cells of day 7 (FL5), 14 (FL3), and 21 (FL1) whereas the second row shows PKCαKR cells of day 7 (FL5), 14 (FL3), and 21 (FL1).

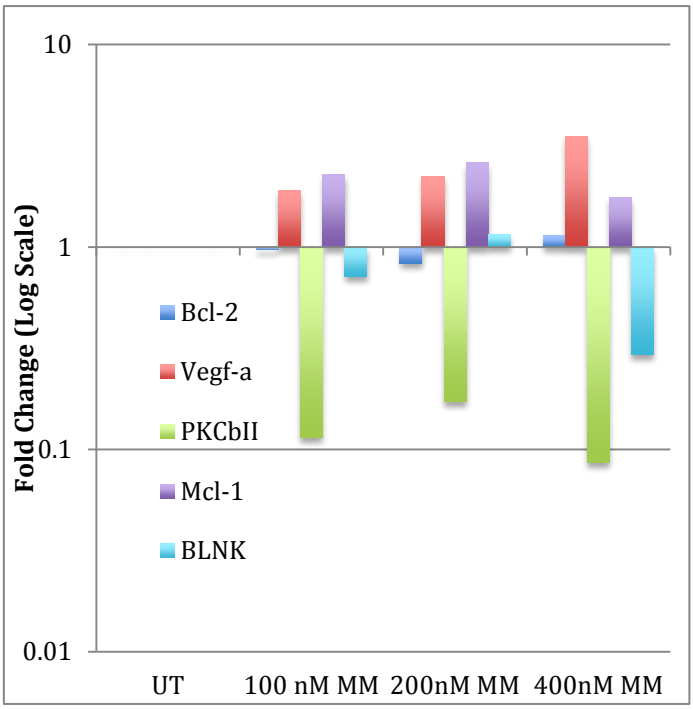
b) CD19+ cells (bottom right box) within the GFP+ cells, also the box on the top left shows myeloid cells (CD11b+). The distribution of the graphs is the same as in a).

c) CD19+ cells tagged with CD23 vs CD5 MIEV cells (top row) and PKCαKR cells (bottom row) of days 7 (FL5), 14 (FL3), and 21 (FL1) respectively. The distribution of graphs is the same as in figure a).

d) Western blots of untreated samples of PKCαKR protein lysates of day 10, 17 and 24 respectively. Top: protein expression levels of PKCβII, Bottom: β-Tubulin levels in the same samples, as a loading control.




a)

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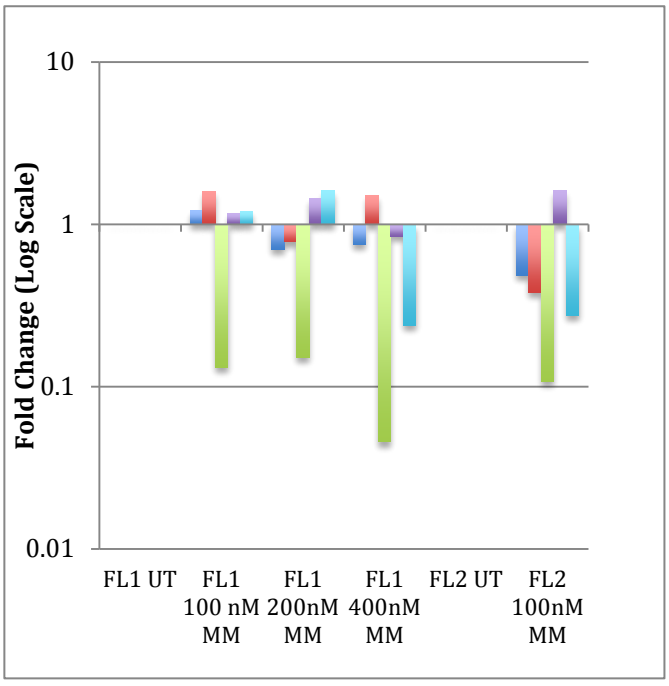


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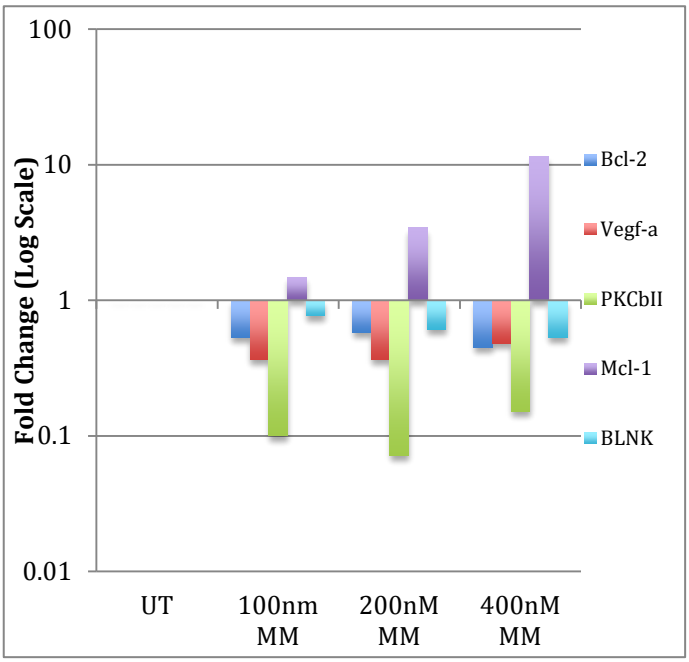



c)

Figure 2: PKCαKR cells treated with different concentrations of Mithramycin (a Sp protein family inhibitor) and the resulting expression of PKCβII and other genes seen in the graph.

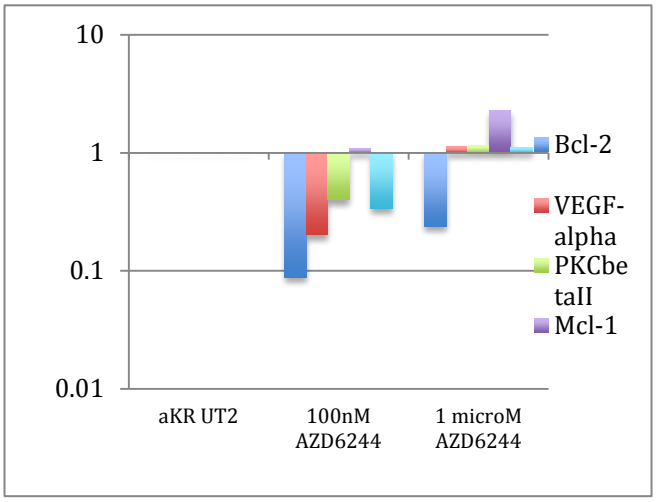
a) Day 23 cultures of PKCαKR cells are shown from two different replicates FL2 and FL3 treated with 200 nM MM, and one sample of FL3 treated with 20% FCS (serum).

b) PKCαKR cells from an additional biological replicate FL1 cells treated with MM

c) Pre amplified PKCαKR cells from FL1 and FL2 mice treated with MM

d) Pre amplified data of FL2 set of mice cells treated with MM.

a)

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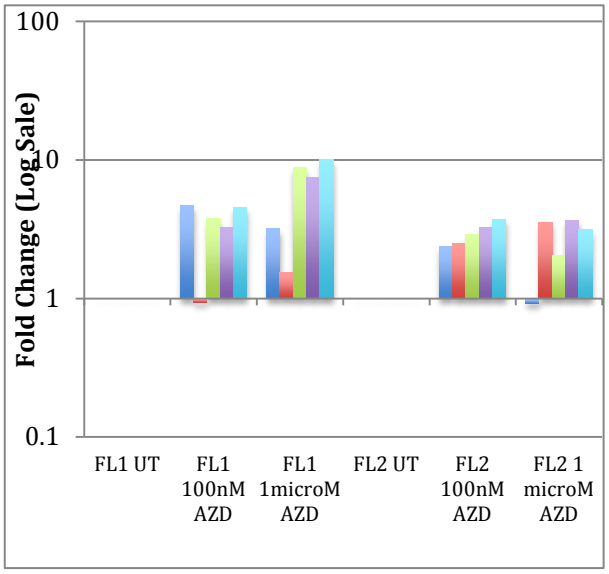


c)

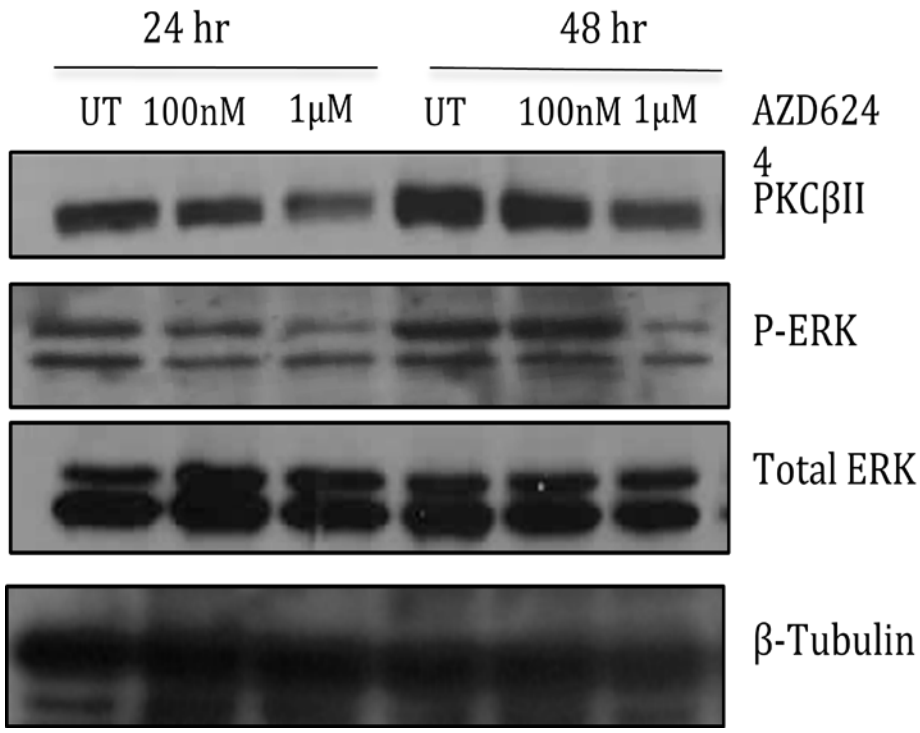


Figure 3: Expression of PKCβII upon treatment with the MEK inhibitor, AZD6244.

a) PKCαKR cells were treated with 100 nM and 1 μM AZD6244 and the resulting gene expression levels.

b) PKCαKR cells from both FL1 and FL2 mice FL were treated with 100 nM and 1 μM AZD6244 and resulting gene expression levels.

c) Western blots of PKCαKR protein lysates were treated with AZD6244 for 24 hr (bands 1-3) and 48 hr (bands 4-6). The blots show PKCβII, phospho-ERK levels, total ERK and β-Tubulin protein expression levels. β-Tubulin is the loading control for PKCβII, and total-ERK is the control for p-ERK.

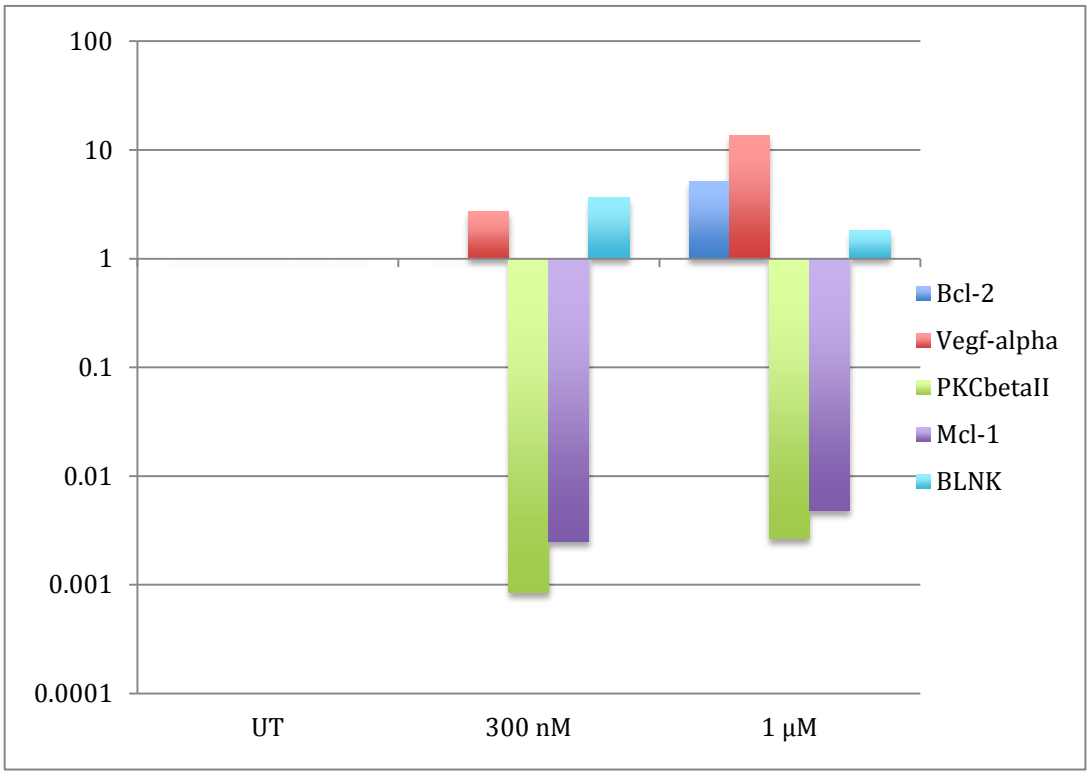


Figure 4: Control cells (MIEV) treated with the drug 5-azacytidine to induce hypomethylation and the resulting gene expression genes including PKCβII.