**ENSAT Short Visit Report – Ian Rose, 2nd-16th March 2013**

**Visit to:**

Professor Enzo Lalli’s group,

Institut de Pharmacologie

Moléculaire et Cellulaire

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***Electroporation for targeted knock-in and knock-down in the adrenal cancer cell line NCI-H295R***

The human adrenal cortical cancer cell line NCI-H295R is one of the most commonly applied models to study cellular adrenal biology *in vitro* and importantly expresses all crucial enzymes involved in human adrenal steroidogenesis*.* The research group of Professor Wiebke Arlt has a strong research focus on the regulation of steroidogenesis, in particular androgen biosynthesis, via steroid sulfation by the sulfotransferase SULT2A1. We have discovered inactivating mutations of PAPS synthase 2 (PAPSS2), a crucial co-factor of the DHEA sulfotransferase SULT2A1, as a novel cause of androgen excess. We are now focusing on the role of the two PAPSS synthase isoforms – PAPSS1 and PAPSS2 in the regulation of steroid sulfation in adrenal tumors, analyzing steroid output and proliferation.

To study the differential effects of PAPSS isoforms 1 and 2 *in vitro*, an overexpression/knock-in accompanied by the mirror-imaged knock-down approach would provide useful insights into their respective physiological role. However, NCI-H295R cells have proved to be extremely difficult to transfect by lipofection since Polyplus Transfection replaced their transfection reagent JetSI-Endo with INTERFERin. I have tried to optimize several different transfection reagents including Qiagen’s HiPerFect Transfection Reagent, Invitrogen’s Lipofectamine 2000, and Biontex’s Metafectene, and it seems that there is currently no reagent available that achieves satisfactory transfection efficiencies. Electroporation would provide an alternative and has been successfully established by the research group of our collaborator and host of this project, Prof Enzo Lalli, working closely with Lonza to develop a protocol that achieves high transfection efficiencies. **I visited the lab of Professor Enzi Lalli to learn electroporation as a means of transfecting NCI-H295R cells to subsequently establish this method in Birmingham.** Establishing nucleofection in the laboratory of Professor Wiebke Arlt would not only contribute to the elucidation of PAPSS isoforms in this particular project, but also provide an important tool for further research when investigating other adrenal proteins of interest.

**Work carried out during visit**

During the visit my primary aim was to get hands-on experience with the optimized AMAXA Nucleofection protocol. Upon arrival, I began working on the nucleofection of the NCI-H295R cells using a protocol that had been optimized for the efficient knockdown of FATE1, a gene of particular interest to the Lalli research group. Following cell culture of NCI-H295R cells I used nucleofection to transfect the cells with siRNA’s for PAPSS2 and SULT2A1 (DHEA Sulfotransferase) as positive controls as they have previously been used in our group to generate >80% knockdown of their targets genes in NCI-H295R cells when using the now unavailable JetSI-Endo transfection reagent. Along with the positive control siRNA’s, I also completed nucleofection of a negative scrambled siRNA control (SC) and 3 siRNA’s for BPNT1 (bisphosphate nucleotidase 1) a gene involved in the regulation of DHEA sulfation by converting PAPS to APS.

I concentrated on identifying the optimal time point by harvesting mRNA at 24, 48 and 72 hours to assess siRNA knockdown efficiency using quantitive PCR (QPCR). I collected mRNA from the cells nucleofected with siPAPSS2, siSULT2A1, siBPNT1 (1, 2 and 3) and SC using the Qiagen RNeasy kit at each time point. Following DNase treatment and cDNA synthesis, the expression levels of BPNT1, PAPSS2, SULT2A1 and 18s genes were measured using TaqMan gene expression assay for each nucleofection to see if I could replicate previous data generated using JetSi-Endo, which appeared to reveal a connection between the expression levels of all three genes.

**Description of main results obtained**

Preliminary data from our group has shown that in NCI-H295R cells siRNA of PAPSS2 results in the increased expression of SULT2A1, where conversely the siRNA of BPNT1 results in the decrease in SULT2A1 expression.   
When looking at the optimal time point for siRNA knockdown of PAPSS2, SULT2A1 and BPNT1, I had a problem with the yield of mRNA from samples collected at 24hrs meaning I was unable to assess the knockdown at this time point (AMAXA manual suggests that 24hrs is the optimal point in most cases). Also, using the TaqMan 18s endogenous control caused an issue where I was unable to measure a Ct value for a control to allow me to calculate the change of the expression levels of the genes of interest according to the deltadeltaCt method. However, when directly comparing the Ct values for each gene following siRNA nucleofection after 72 hours, it appears that there is a reduction in the expression of the target gene as indicated by an increased Ct value (Fig.1) potentially indicating that the siRNA is having an effect. siBPNT1 1 and 3 both show a ~ 0.5 Ct increase indicating a potential reduction in expression of ~25% (while siBPNT1 2 had no effect). Similarly, siPAPSS2 shows a ~ 0.75 Ct increase, which is ~37.5% reduction, and siSULT2A1 shows >1 Ct increase indicating a >50% knockdown efficiency.

**Figure 1.** Comparison of Ct values of BPNT1, PAPSS2 and SULT2A1 from duplicate nucleofections of the 6 siRNAs after 72 hours. The bars highlighted in red represent the siRNA target gene. siBPNT1 1, siBPNT1 3, PAPSS2 and SULT2A1 all appear to cause an increase in the Ct value of their target gene indicating a decrease in expression levels.

**Future work, projected publications / articles resulting or to result from the grant**

siRNA-mediated knock-down is an important tool for this particular project on steroid sulfation, but also a methodology we crucially require to explore other interesting target genes we are currently working on as parts of other projects in our group. Therefore, this technique will allow us to explore the effects of different target genes in the most commonly applied model to study adrenal steroidogenesis, the NCI-H295R cell line.

Part of the present project is to compare overexpression and knock-down on different PAPSS isoforms in order to elucidate impact on steroidogenesis, in particular DHEA sulfation. Previously, we have successfully overexpressed PAPS synthase isoforms 1 and 2 and observed differential effects on DHEA sulfation in HEK293 as assessed in functional *in vitro* assays. Now, as a mirror-image approach, siRNA mediated knock-down experiments in NCI-H295R cells are crucial as they complement the overexpression studies. Once established, a publication is imminent as they provide the last and important final series of experiments adding on to data we have already generated over the last few years.

Finally, if the effects of siRNA-mediated knock-down we have previously seen can be reproduced, further investigations looking at other regulated transcripts by micro-array analysis as well as searching for potential interaction partners of PAPS synthases by proteomic studies are imminent.

**Other Comments**

I would like to thank the European Science Foundation for granting me funds to allow me to visit the research group of Professor Enzo Lalli. I would also like to thank Professor Enzo Lalli for the opportunity to visit his group, and also all members of his group for their support and assistance during my visit in particular Dr Mabrouka Doghman.