JAMES RENNIE BEQUEST

REPORT ON EXPEDITION/PROJECT/CONFERENCE

Expedition/Project/Conference Title: Role of RPS19 in Diamond Blackfan Anaemia.....

Travel Dates: 01/06/06-22/08/06.....

Location: Washington University, St Louis, MO .....

Group Member(s): Emma Hall.....

Original Aims: To study the expression of the small ribosomal protein 19 in a cell line model of erythropoeisis, G1E-ER4. However cells did not grow well.
Aims: To produce a system for study of the effect of Diamond Blackfan Anaemia patient mutations in the RPS19 gene when expressed in the human cell line HeLa. .....

OUTCOME (not less than 300 words):-

Diamond Blackfan Anaemia (DBA) is a rare congenital aplastic anaemia. Patients display low red cell counts, but normal levels of other blood cells. In addition some patients show other congenital abnormalities including craniofacial malformations and thumb abnormalities. In 25% of cases mutations in the gene encoding the small ribosomal protein 19, RPS19, are responsible for the disease. However the molecular mechanisms and pathophysiology are not well understood.

My original project was to investigate why a defect in a ribosomal protein which is ubiquitously expressed caused a disease specifically in red blood cells by studying erythropoiesis in the cell line G1E-ER4, an mouse immature erythrocyte cell line that can be induced to undergo differentiation by adding tamoxifen or estradiol. However this cell line proved difficult to grow so a new project was designed.

The aim of this next project was to design a system to study the effect of patient mutations in RPS19 on HeLa cells, a human epithelial cervical cancer cell line. The overall aim of the project is to transfect HeLa cells with a construct containing the RPS19 gene, tagged to distinguish it from endogenously expressed RPS19 and containing silent mutations meaning it is resistant to knockdown by RNA interference. The endogenous RPS19 will then be knocked down using siRNA, so the cells only express the mutant RPS19. This will then be repeated once various DBA patient mutations have been introduced into the RPS19 gene. The knockdown will be confirmed by Western blots and then the effect these mutations have on the cells will then be analysed by looking at ribosomal profiles and examining ribosomal biogenesis using Southern blots.

My role in this project was to design and make constructs containing the siRNA resistant, tagged RPS19 gene and transfect these into HeLa cells.

To begin this project I first designed primers to use for RT-PCR to amplify the RPS19 cDNA from human RNA. These primers introduced a 6-histidine (6-HIS) tag on either the N or the C terminus of the protein and an EcoR1 site at the C terminus and an Age1 site at the N terminus of the gene. I made constructs with tags at both ends of the gene in case the tag interfered with the protein function at one end. I amplified N-HISRPS19, C-HIS-RPS19, and no tag-RPS19 and cloned them into the plasmid, pIRESneo3. This contains an amp^r gene so we transformed ultracompetent cells and plated on plates containing ampicillin to select for the cells containing the plasmid. I then performed minipreps and maxipreps on these to produce large amounts of DNA and sent this for sequencing to verify I have the correct inserts.

I then performed targeted mutagenesis at the siRNA site to create silent mutations making siRNA resistant RPS19. I designed a primer with 3 silent point mutations at the site the siRNA targets and another primer downstream of the RPS19 gene in the plasmid and used these to amplify a fragment with the siRNA mutations. This was then cut with Not1 and inserted back into the original vector cut with Not1 and sequenced to confirm the insertion was in the correct orientation.

I also attempted to transfect the constructs before mutagenesis into HeLa cells to try work out a protocol to transfect the cells. I used electroporation to transfect HeLa cells with each of the constructs, a control electroporated with no DNA and a positive control transfected with pGKneo, a plasmid expressing just neo. These were plated onto large Petri dishes and the medium changed to include G418 after 24 hours. Neo resistant colonies appeared in the positive control with pGKneo after several days, showing the electroporation worked, and all the cells in the control with no DNA transfected died showing the G418 worked well. However no neo resistant colonies appeared in the HeLa cells transfected with my constructs. This could be because there is some problem with the expression of the neo gene, a failure of electroporation to get the constructs containing RPS19 into the cells, or perhaps overexpression of the RPS19 gene is lethal to the cells. One plan to overcome this problem is to try lipofection - another method to get the DNA into the cells. I set this up but there wasn't time to analyse the results during my stay.

The constructs I have designed and made are vital to this project and will allow the study of the effect of patient mutations in RPS19 on ribosome biogenesis and function. This may offer valuable insights into the molecular causes for DBA, perhaps leading to possible new treatments or cures, and maybe to a greater understanding of ribosome biogenesis and function in general.

This project has help me gain a better understanding of how a research laboratory works, and has taught me skills in molecular biology including PCR, cell culture, cloning and transfection. This has helped me decide that scientific research is the career path I would like to pursue, allowed me to see how better to achieve this and given me valuable experience in this field.

This trip also gave me the opportunity to live and work in the USA for 3 months, experiencing a different culture and exploring St Louis. I also travelled to Chicago for three days and Kansas for a weekend, both of which I thoroughly enjoyed.

I would like to thank Philip Mason and Monica Bessler for allowing me to come to their laboratory to work and welcoming me into their home, Rachel Idol and Sara Robledo for allowing me to work on the DBA project with them, and all of them for teaching me the skills necessary for the project.

I would also like to thank the James Rennie Fund for making the whole trip possible for me – it has been an enjoyable and beneficial way to spend my summer.

Photos:

Buckingham Fountain, Chicago

The Gateway Arch, St Louis