Investigations into the roles of Wnt/beta-catenin pathway in retinal development and degeneration.

Background

Signalling between cells by secreted soluble protein growth factors is essential for maintaining the function of normal tissues. Previous studies by us and other research groups have shown that a growth factor signalling pathway (Wnt/beta-catenin signalling pathway) is present and active in both the developing and adult eye. In certain retinal diseases, particularly degenerative disease of the retina, the levels of various components of this pathway are changed. This suggests that this pathway or its activity is changed in retinal disease. However, it is not known whether changes in this signalling pathway cause or contribute to the disease or whether they are changed as a consequence of the disease and reflect attempts by cells to compensate for degenerative processes. Studies of this pathway in other organs, tissues and cells indicate that it plays crucial roles in maintaining functional stem cell populations, particularly during development of the embryo. Stem cells are important cells in any tissue as they are the cells that have the capacity to replace damaged or lost cells and have the potential to be used in therapies. The overall aim of this project was to develop techniques that would allow us to investigate the role of the Wnt/beta-catenin signalling pathway in the developing and adult retina.

An important tool in the study of how genes function is to be able to manipulate the activity of genes in cells, tissues and organs of animals. The best model for doing this in living mammals is the mouse. 'Gene knockout' approaches in mice have provided powerful tools to alter the function of genes in the whole animal. However, one problem with the normal gene knockout approach is that the gene is knocked out (non-functional) from the earliest stages of development of the embryo. If the gene under study is involved in very early stages of development, then the embryo can die before the eye and retina forms and it is then not possible to study the function of the gene in the eye or retina. An important advancement in knockout technology is the Cre-LoxP approach, which permits a gene to be deleted or mutated in a specific tissue or at a specific time by using a modular system, comprising the Cre recombinase enzyme and its specific DNA binding site (LoxP sites). These two modules originally were found in bacteria and don't normally occur in mammals. If the two components are present in a cell, the Cre enzyme binds the LoxP DNA sites and then cuts and recombines the DNA, thereby deleting the DNA between two LoxP sites. These two components (Cre and LoxP sites) can be inserted into the DNA of mice and on their own do not affect the mouse or its development. However, when we mate the mice that carry each of these components, then the offspring will carry copies of both components (Cre and LoxP) and the offspring will have cells with deletions or mutations of the genes that were flanked by the LoxP DNA sites. By manipulating where Cre is produced, we can mutate or delete a gene in the tissue of interest (retina), without affecting the viability of the offspring.

A critical determinant for this technique is the availability of mice that express Cre in a tissue-specific manner. In particular, there are few lines of mice that express Cre in unique and distinct patterns in the retina that permit the study of gene function in the retina. We have recently generated mice that expressed Cre in the developing retina.

The first aim of this project was to characterise the patterns of Cre activity in our two lines of transgenic mice (MLR34, MLR37) to see if these could be used to delete genes in the retina.

The second aim was to whether the Cre expressed in the retina of these lines could actually delete a functional gene in the developing mouse retina. Beta-catenin is a central component of the Wnt signalling pathway and its deletion will inhibit the pathway. It is a gene that we have successfully knocked out in other parts of the eye and thus we have many of the tools needed to study this gene in the retina. If we can show that the Cre lines will delete the beta-catenin gene then we know that we have useful Cre lines with which to study this and numerous other genes in the retina.

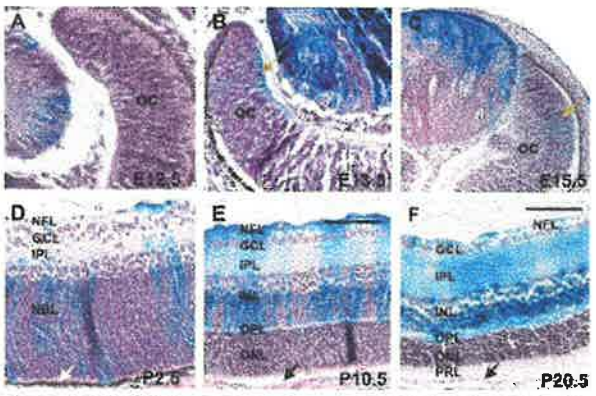

Figure 1. Expression of Cre (blue) in retinae of F1 bCat34 mice at E12.5, E13.5, E15.5, P2.5, P4.5 and P10.5. The expression of Cre increases with age and is predominantly in the inner retina

Use of the grant funding

The Retina Australia grant provided funding for a part-time (50%) salary for a post-doctoral researcher (Dr Maria Kokkinos) and resources (animal costs, antibodies and other reagents) to complete experiments in Dr de Iongh's laboratory. The rest of Dr Kokkinos' salary was provided by a Melbourne University (MRGS) grant to work on a related project on the lens.

Aim 1: Characterisation of transgenic mice that express Cre recombinase in the eye. Initial studies of the MLR34 and MLR37 mice suggested that there was significant expression in the newborn retina but this had been inadequately described. To address this we carried out more detailed studies. We mated MLR34 and MLR37 mice with "reporter mice" and studied the embryos. The reporter mice have a special LoxP-flanked transgene in their DNA that responds to the presence of Cre by producing an enzyme (b-galactosidase), which can be stained with a stain called X-Gal (Figure 1). Whenever a cell has Cre and the reporter, the cell will produce the b-galactosidase enzyme and can be stained blue with X-Gal. This technique allowed us to track where Cre is active during development of the retina. It is important to know where Cre is active as this is where a gene will be deleted if we

produce mice that express Cre and have a gene flanked by LoxP sites. Figure 1 shows the results of this kind of experiment; the blue stain in sections of the retina shows where Cre is active during development of the retina in the mouse embryo and in postnatal mice. The important thing to note is that the blue stain is present in columns of cells through almost all layers of the retina (Fig 2D-E), indicating that we can knock out the gene in some regions of the retina but not others (variegated pattern). This may be useful as it would allow us to compare, in the same retina, groups of cells with a gene knockout to cells that are normal.

In summary, these results show that in the retinae of MLR34/37 mice there is significant expression of Cre, which has the potential to cause gene deletions at LoxP sites in the DNA of cells in the retina. A disadvantage of the variegated expression is that the analyses of these mutant retinae will require careful study of which particular cell in any preparation has Cre expression and is thus will have a gene deletion (mutant). An advantage is that mutant and wild-type cells can be studied side-by-side in the same retina.

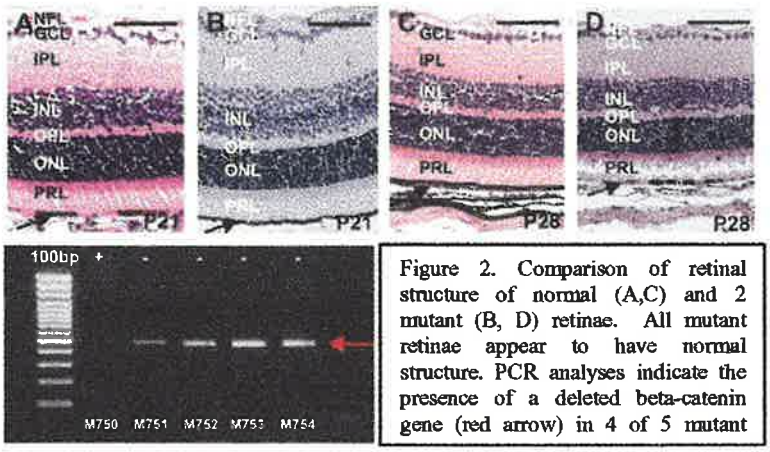

Figure 2. Comparison of retinal structure of normal (A,C) and 2 mutant (B, D) retinae. All mutant retinae appear to have normal structure. PCR analyses indicate the presence of a deleted beta-catenin gene (red arrow) in 4 of 5 mutant

Aim 2: Conditional deletion of the beta-catenin gene in retinae of transgenic mice.

To investigate whether the levels of Cre in these mice are sufficient to actually delete a gene in the retina we mated the Cre mice (MLR34 and MLR37) to mice that have the beta-catenin gene flanked by loxP sites. While this requires 2-3 generations of breedingwe were able to identify the mice that actually have a deleted gene by taking DNA from the retina and carrying out a polymerase chain reaction (PCR). Figure 2 shows an example of a PCR of retina DNA from 5 mice, of which 4 have a deleted gene (white bands, red arrow).

The structure of the retina in these mutants is normal. Figure 2 shows the retinae of two mutant eyes (B, D), which are very similar to normal eyes (A, C). This may mean that the deletion of beta-catenin had no effect on retinal development or that the effects were subtle and difficult to see. It may also mean that the levels of Cre are not high enough or extensive enough to delete the gene in a lot of cells. Since the PCR analyses showed that the Cre had effectively generated at least one gene deletion in these retinae it was possible that one functional copy of the gene still remained in many or all of these cells (each cell has two copies of a gene). If both copies of the gene are deleted then there should be little or no beta-catenin present in cells.

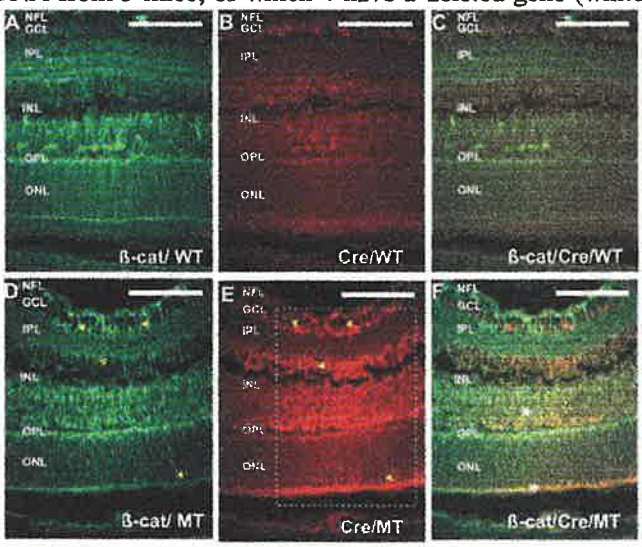

Figure 3. Deletion of beta-catenin by Cre in mutant (MT) mice. Double immunocalisations of beta-catenin (green) and Cre recombinase (red) in control (A-C) and mutant (D-F) retinae. In merged images (C, F) deletion of beta-catenin is evident in some cells (orange-red stain) of the mutant retina (F). WT=normal mouse.

To investigate this we used an antibody to label the cells that have Cre (in red) and another antibody to label the beta-catenin (green) (Figure 3). This experiment showed that we had in fact deleted beta-catenin in some cells. However, the deletion is not very extensive. This means that the mice may be useful for generating mutations in a small group of cells in the retina and then studying these cells next to normal cells in the same retina.

Future Studies

Our future studies are aimed at increasing the efficiency of the Cre activity in the retina in our mutant mice by breeding experiments and thus generate more extensive gene deletions in retina. We also aim to study more closely the effects of deleting the beta-catenin gene in the developing eye.

This Retina Australia Grant has allowed us to test two novel Cre lines and was invaluable as a bridging support. We are now funded by an NHMRC grant and we intend to further extend and develop these studies under the umbrella of that grant. These studies are being presented at the ARVO meeting in Ft Lauderdale in May 2006.