**Role of a novel miRNA in the dominant syndromic disorder of macular dystrophy and split hand and foot malformation**

Professor David M Hunt and Professor Shaun P Collin, School of Animal Biology, UWA Oceans Institute and Lions Eye Institute, University of Western Australia

**Final report**

The project has focussed on a syndromic form of macular dystrophy in a French family where the retinal defect is inherited in a dominant fashion along with split hand and foot malformations (SHFM). A review of the literature relating to different forms of SHFM reveals that in many cases, chromosomal deletions are causative, leading in many cases to haploinsufficiency for genes within the deleted region. In order therefore to see whether such changes are present in our family, we looked directly at the genome (using microarray analysis) to identify regions that show evidence of deletion. This analysis revealed one such a region on chromosome 5p15.33 that covers approximately 180 kB of DNA. The presence of a deletion was confirmed independently (using the technique of quantitative polymerase chain reaction (PCR)).

The deleted region is gene-poor with a single annotated protein coding sequence of unknown function, a processed transcript, a sequence which is annotated as a novel non-coding or microRNA (miRNA), and a so-called long non-coding RNA (lncRNA). miRNAs are short RNA molecules with an average length of 22 nucleotides that act as regulators of gene expression by binding to complementary sequences in mRNA transcripts and thereby blocking (or silencing translation and the production of the protein product. miRNAs. lncRNA on the other hand are long transcripts that are again invovled in gene regulation, although howthey do this is still uncertain.

The main aims of the project were (i) to confirm the linkage of the disorder to chromosome 5p15.33, (ii) to determine whether the identified protein coding region and the processed transcript within the deleted region are expressed within the retina, (iii) to determine whether the potential targets for the putative Chr 5 miRNA are expressed in the retina, and (iv) to determine whether the putative Chr 5 miRNA and the lncRNA are transcribed in the retina.

**Human retinal RNA**

RNA was extracted from human retinae dissected from eyes donated to the Western Australian Eye Bank after obtaining appropriate ethical permission. In addition, a small sample of human retinal RNA was purchased from Clontech.

**Linkage analysis**

By using single nucleotide polymorphisms (SNPs), we have been able to show that the disorder maps to one of two chromosomal regions, either the region of chromosome 5 that harbors the deletion in affected individuals or to a region of chromosome 9. In order to confirm the linkage, we intend to extend the SNP analysis to a new affected member of the family.

**Expression of the identified gene sequences within the deleted region on chromosome 5**

PCR was used with determine whether the protein coding region and the processed transcript are expressed in the retina. Complementary DNA (cDNA) was synthesized from retinal RNA and used as a template with primers designed to suitable regions of the two target sequences. A number of primer combinations were used but all failed to generate amplicons. From this, we concluded that neither region was active in the production of RNA transcripts in the retina, so it is unlikely that haploinsufficincy for either of these genes is responsible for the disorder.

**Identification of expressed target genes**

miRNAs generally target sequences in the transcribed RNA sequence of expressed genes. Five genes were identified that show complementary sequences to the putative Chr 5 miRNA. PCRs

with primers designed to the coding regions of these genes were again used with retinal cDNA as template alongside test amplifications using human genomic DNA as template. Amplified fragments were obtained with genomic DNA and these were sequenced to confirm identity, but no fragments were obtained from PCRs using retinal cDNA. We conclude from this that none of the target genes are expressed in the retina. A search of the database of expressed sequences confirms that transcripts from these genes are not been recovered from eye or retinal tissues.

**Transcription of the putative Chr 5 miRNA in the retina**

The putative Chr 5 miRNA shows close homology to a family of miRNA sequences designated miR-548. The miR-548 family is primate-specific, with many copies present in the human genome. None however have been mapped to the deleted region of chromosome 5.

RNA samples were prepared from human retinal tissue using a technique (the *mir*Vana™ miRNA isolation system from Ambion) that ensures the efficient recovery of small RNA molecules. The detection of novel miRNAs is not however straightforward, as conventional PCRs with primers complementary to the target sequence cannot be used to amplify such short sequences. To overcome this difficulty, we used a system designed by a commercial company (Qiagen - PCR miScript system) that uses a primer specifically designed to amplify the putative Chr 5 miRNA if present. The experiment failed to yield any product in repeat PCRs.

To explore this further, we decided to take advantage of the recent technological advances referred to as Next Generation Sequencing (NGS) to sequence all expressed small RNA molecules in the human retina (ie to obtain a so-called transcriptome). In this way, we were confident that if the putative Chr 5 miRNA was transcribed, then we would find it amongst the sequences of the transcriptome. The NGS was carried out by the Beijing Genetics Institute on RNA isolated from two retinae and generated a dataset that contained to several million sequences. Of the 853 miRNAs that are now known, 269 are expressed in the retina, with most of the miR-548 family members present. However, a search of the data failed to identify the putative Chr 5 miRNA.

**Transcription of lncRNA**

In the absence of any clear evidence that the the putative Chr 5 miRNA is expressed in the retina, we have now turned out attention to the lncRNA identified in the deleted region. Again, working with such RNAs is not as straightforward as working with “normal” coding genes as the direct of transcription is generally not established and the transcripts undergo multiple alternative splicing. We are still in the process of examining this lncRNA using multiple combinations of PCR primers.

**Outcomes**

From our study, it would appear that the putative Chr 5 miRNA that is present within the deleted region on chromosome 5 in affected members of the family with macular dystrophy with SHFM is not transcribed in the retina. It is unlikely therefore that this disorder arises from haploinsufficency for this putative miRNA. This leaves the very real possibility that a lncRNA is involved in the disorder. If so, this would be the first case where a retinal disease has such an underlying cause. Another macular dystrophy that we are continuing to study is the so-called North Carolina macular dystrophy, named after the location in the USA of the original family. This disorder maps to chromosome 6q14-q16.2 and, as for the macular dystrophy with SHFM, all attempts to find mutations in a coding gene have failed. We now suspect that this disorder may also arise from a mutation in a lncRNA.

To our knowledge, this is the first time that a small RNA transcriptome has been generated from human retinal tissue. Given the known role of miRNAs in AMD, these transcriptomes will provide a very significant resource for the study of miRNAs in retinal disease and the generation of two transcriptomes in parallel will permit some estimate of variability in expression. A full analysis will be carried out and the results prepared for publication.