Investigating the potential regulation of Asic2 by microRNAs

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Abstract

Multiple Sclerosis (MS) is an inflammatory disease which causes neurodegeneration. It is a disease which affects mainly young adults, and symptoms become more aggressive over time. It is thought that mutations in a variety of genes may be a large contributor to the development of MS. Many Linkage and Genome Wide Association Studies have been performed to find genes which may be mis-expressed among different sections of the world's population. Through one of these Genome Wide Associations a Single Nucleotide Polymorphism (SNP) was found in the 3' UTR of the ACCN1 gene. This gene codes for Acid Sensing Ion Channel 2, a major ion channel predominantly found in cells within the Central Nervous System (CNS). As this channel is closely connected to systems in the CNS which are known to be affected in patients with MS, it was thought that the SNP should be investigated further. As the SNP was found in the 3' UTR it was suggested that miRNAs may play a role. This study took on this suggestion and investigated the possibility of ACCN1 being controlled by a miRNA and whether this control was in any way affected by the SNP found.

The initial experiment found that of the two variants, rs28936A was controlled more strongly by a miRNA than the variant rs28936G. Resequencing of the samples showed a further three SNPs in the same region. Several miRNA target sites were predicted that contained at least one of the SNPs, however none of these could be validated experimentally using luciferase essays. The three SNPs were tested individually using the same luciferase experiment but none were found to have a more significant effect over luciferase expression over the others. The results therefore indicate that all SNPs contribute to the reduction in miRNA binding. Further research is proposed to investigate in more detail, potential miRNA candidates. It was concluded from these results that the ACCN1 gene is being controlled by an unknown miRNA via binding to the 3'UTR, that the rs28936A variant is more strongly controlled by the miRNA and that of all SNPs found (including the two originals and three 'new' SNPs) none have a significantly stronger miRNA control than the other.

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Chapter 1: Introduction

Multiple Sclerosis pathology

Multiple Sclerosis (MS) is an inflammatory disease which causes neurodegeneration, leading to impaired motor and cognitive neural functions (Bernardinelli. L et al. 2007, Sawcer. S et al. 2005, Hafler. D et al. 2007, Haines. J et al. 1996, Haines. J et al. 1998, Baranzini. S et al. 2009, Comabella. M et al. 2008, Ban. M et al. 2003, Bahlo. M et al. 2009). It is thought to be an autoimmune disease due to the immune system attacking self cells in the Central Nervous System (CNS), however it is set apart from other autoimmune diseases as it develops at a later stage in life (early adult). It is generally accepted that it is a mix of genetic and environmental factors which contribute to the onset of the disease; however the direct trigger is unknown (Figure 1). It is the most common form of neurodegenerative disease, which results in both axonal and myelin degeneration due to a dysfunction of the T cells, in the central nervous system during the younger years with relapse and remission periods throughout the patient's life. The subsequent result of the loss of axons and myelin is the formation of plaques in the CNS. These plaques accumulate over time and participate in the worsening of neural function. There are different levels of severity based on the pattern of relapses, which can range from instant neurological damage with no remission period, to slow degeneration over time with remission periods and increasing severity. Symptoms of MS include impaired memory, learning difficulties, speech impairment, impaired attention span, problems processing information quickly and physical difficulties. Currently there are no treatments for the direct cause of MS as this is still unknown; however there are many drugs which are used to treat the symptoms.

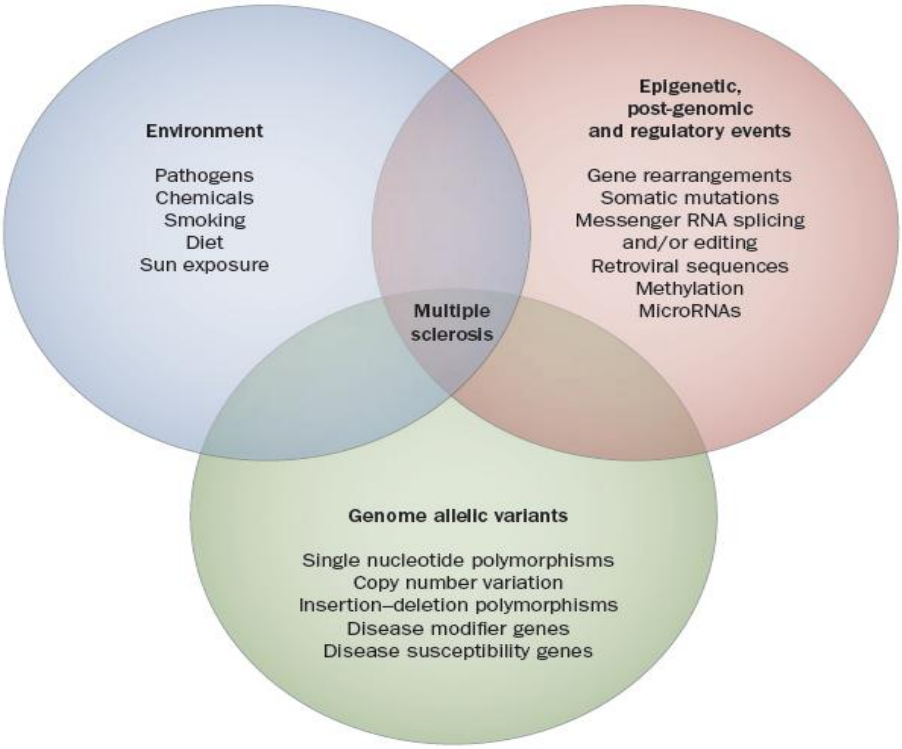


Figure 1: Diagram showing some of the suggested and suspected causes of Multiple Sclerosis. It is suspected that a combination of many if not all of these factors are implemented in the development of MS (Oksenberg. JR et al. 2008).

Current Treatments

Drug therapy is the foremost treatment widely available to date. However many of the available drugs are aimed at preventing the development of symptoms, not to directly treat the underlying problems. This has been mainly due to our previous lack of understanding as to the most prominent causes of MS. However with our current developments more evidence has come to light as to the major components of MS. Previous treatments such as Interferon beta which alters antigen presentation to reduce phagocytosis and alters T cell proliferation, cytokine and chemokine expression patterns, Glatiramer Acetate (GA) (which is a pool of peptides which alters T cell differentiation specifically by enhancing Th2 polarised T cells) and Natalizumab (which is a monoclonal antibody directed against the late activated antigen-4 which inhibits lymphocyte migration through the blood-brain barrier to the CNS) (Buck. D and Hemmer. B 2011, Comabella. M and Vandenbroeck. K 2011), are all designed to prevent the advancement of secondary effects, caused by the primary problems. They are effective however, the level of effectiveness varies among patients. A new technique which aims to understand the beginning steps of the disease utilises the latest genetic screens (which are more sensitive and accurate than previously) and will help indicate the primary problems causing the onset of MS on a patient to patient basis. This

allows appropriate treatment to be administered to the appropriate primary effect as evidence has strongly suggested there is more than one. Use of Biomarkers (molecules that are affected due to disease or infection and indicate the affliction) have also been shown to indicate the effect of drug therapies on different patients in studies on a small scale. These could potentially be used as indicators of the effectiveness of drug therapies (looking at the change in their expression in individual patients), in order to give the most effective treatment possible. This may lead to individual doses of effective drugs depending on the patient's reaction to the drug (Buck. D and Hemmer. B 2011, Comabella. M and Vandenbroeck. K 2011).

Linkages and Genome Wide Association Studies

Many studies have researched for the genetic components for MS using two major experimental designs to determine these mutated genes. The first experimental technique which has been used for MS is a genetic linkage. This type of study determines the degree of linkage between mutations which have been found to contribute to MS. Genetic Linkage is the probability that a set of genes along a chromosome will be inherited together. The higher the degree of linkage, the more likely that the genes will be inherited together, which would suggest that the genes are all involved in the onset of MS. This technique uses a high volume of samples which are collected criteria such as age of disease onset, severity of disease and progression of disease. The results obtained from this technique can be used to help us to find which genes or areas of chromosomes are commonly occurring in MS. Microarrays can be used in linkage studies to indicate how close the genes are along the chromosome, whether the genes are likely be replicated together and whether the distances occur frequently in MS suffers, however it is not necessary to do this unless there is a large number of samples.

An early study in 1996 by the Multiple Sclerosis Genetics Group conducted a complete genomic screen (linkage analysis) for MS (Haines. J et al. 1996). In this screen 52 families with multiple affected generations, 443 markers (areas on the genome which are known) across all of the chromosomes were tested for linkage for MS. As it can be expected from a random search, of these markers only 19 were found to have any linkage. One of the loci found to have high linkage was the MHC region on chromosome 6 and was the only one which was previously known to have a genetic effect. There was however no outstanding evidence to suggest linkage in the other genes found in the study. A follow up analysed an additional 23 families to the original set meeting the same criteria, focusing on three markers on chromosome 6 (where the MHC region is found), TNFβ, D6S283 and HLA-DR. TNFβ was found to have moderate to low scores on all three analyses. D6S283 however, showed much higher scores and HLA-DR showed the highest scores

overall. This gave more evidence to suggest the involvement of the MHC region however the results were not positive enough to regard as significant. This suggested that the genetic effect is likely complemented by environmental factors, which working with the mutations, cause the onset of the disease.

Two years later another linkage study was performed by the same group for a more in depth evaluation of the high degree of linkage of the MHC region in MS patients (Haines. J et al. 1998). 98 familial samples were tested to determine whether the MHC region is linked in familial MS patients. The most consistent replicated findings had been of an association with the HLA-DR2 allele which is found in the MHC region however there is also evidence against this. This had led to the suggestion that there are different mutations which associated with familial and sporadic MS. To test for specific allelic associations in the MHC area the HLA-DR2 was used to test for association. The HLA-DR2 allele was shown to have a direct association with familial MS. Three genes in the MHC region (HLA-DA, *D6S273* and TNFβ) also demonstrated a strong linkage to familial MS. Over all, the results from this study suggest that in familial MS the genetic linkage to the MHC region is due to the HLA-DR2 allele. It was also shown that familial and sporadic MS cases share genetic susceptibility and that the MHC region is responsible for between 17-62% of the genetic susceptibility. However, there are also minority familial cases which don't show any MHC association that suggests that there are more susceptible genes to be found.

In 2002, a linkage study was performed in Australia on families of European descent (Ban. M et al. 2002). 54 pairs of affected siblings were selected for the study and 397 markers were screened for linkage. From this linkage four sites were suggested to have linkage and another 18 sites to have potential linkage. Many of the sites found in this study feature in previous studies which gives support to those sites found being susceptibility genes. The four sites found are located on chromosomes 2, 4, 6 and the X chromosome and have further contributed to our understanding of the inheritance of MS. Two of the potential linkage sites were found to be close to genes involved in the body's immunological function, which suggests that these areas should be put under further study, considering the nature of immunological dysfunction related to MS. This study has given stronger evidence for the MHC region being a large contributor to disease inheritance and has also suggested more candidates for further research.

A few years later in 2005, the International Multiple Sclerosis Genetics Consortium performed a high density linkage analysis (Sawcer. S et al. 2005). 730 families of Northern European descent were sampled for this study, and after quality control, 4506 markers were investigated in 2692 individuals. As with all of the previous studies there was a strong linkage in the MHC complex. The results in this study give strong evidence that the allele which has the dominant susceptibility is DRB1*1501 and that if there is a secondary allele in the MHC area, it is unlikely to have much of

an effect overall however no evidence from this study suggests there may be one. There was also a suggested linkage on chromosomes 17 and 5. It was also suggested by analysis that there may be an additional locus on chromosome 19 which is independent of the MHC locus. These results give evidence that there are other susceptibility genes outside of the MHC region which may have a significant effect on the development of MS. This study paved the way for another study performed by the same group using the new genome wide association technique, which will be summarised later.

The second of these experiments is known as a genome wide association study. This technique is newer than the genetic linkage and can process and find more possible target genes faster. This takes large volumes of samples from families that have strong familial MS (usually at least two cases of MS and the rest of the family are also screened for the mutations) and screens all of the samples for genes or areas of the chromosomes which show consistent mutations against control samples obtained from families which do not have MS. This information, depending on what percentage of the test samples contains the mutation, would suggest that the mutations found contribute to the onset of the disease. Genome wide association studies have been used since the availability of rapid genome analysis. Microarrays are loaded with DNA and are used to sort through large pools of samples. This is achieved through hybridisation to the DNA on the array by fluorescently tagged probes made from cDNA. They hybridise to the bound DNA and emit light to indicate that it had bound. If there is no light, then the probe has not bound. This could indicate that there is a mutation at this site which will later be identified by sequence analysis. As technology has advanced, the sensitivity of these studies has become more accurate, and more genes are being identified as potential prerequisites for the onset of MS.

The first of these studies was a global genome wide search for alleles which are associated with MS done by the International Multiple Sclerosis Genetics Consortium, published in August 2007 (Hafler. D et al. 2007). This study is one of the first major studies for MS which utilises this style of analysis. The analysis was achieved by collecting 931 samples from families with at least one case of MS and testing them for association via DNA microarray analysis of 334923 Single Nucleotide Polymorphisms (SNP). SNPs are single nucleotide mutations found throughout the entire genome which can occur anywhere and can, if they occur in genes, disrupt expression. This was done to identify whether there were any SNPs present at high analytic frequencies in the MS patient samples. The study after the first round was further replicated with another 609 samples from the same conditions. The results obtained from this study suggested a high association between the interleukin-2 receptor α, interleukin-7 receptor α and Human Leukocyte antigen (HLA) locus (responsible for a large percentage of the genes needed for immunity) and the development of MS. These findings have large implications for study into MS as they are all involved in immunity.

For example, interleukin-2 is known to regulate T cells that express CD4 and CD25. Interleukin-7 is also known to be used in memory T cell homeostasis, as well as for development of gamma and delta T cells which are some of the first to be seen in inflammatory lesions. As T cells are known to be dysfunctional in MS, this may with further research be a very important find.

Another similar study published in 2008 (Comabella. M et al. 2008) focused on Spanish Caucasians. In this study 242 unrelated MS patients (collected from the outpatient's clinic at Unitat de Neuroimmunologia Clinica) and 242 unrelated control samples (found in the hospital transfusion centre) were run through a similar Microarray analysis searching 500,000 SNPs via a criteria based algorithm to identify new loci for their association with MS. From the SNPs scanned, 320 were selected and genotyped in the first Spanish Caucasian replication cohort. After the first analysis the eight SNPs which showed the highest significance were further genotyped in the US Caucasian replication cohort as a final confirmation for a broader sample. From this study the strongest association found was close to the HLA/DRB/DQA loci (in both the Spanish and the additional US samples), further confirming what previous studies, such as that performed by the International Multiple Sclerosis Genetics Consortium, have shown. However, this study also showed a strong significance for a novel locus on chromosome 13 in the Spanish replication and later in the US replication (SNP rs1327328 and rs7326018) which could also play a role in susceptibility via unknown effects on surrounding genes.

A further study conducted a year later (Baranzini. S et al. 2009) performed another genome wide association analysis from individuals of European descent. In this study 2068 individuals were genotyped and eventually after filtering 978 test samples were used with 883 control samples. Where the previous studies had used the same type of Microarray chip (Affymetrix© GeneChip Human mapping 500K), this study opted to use a Sentrix© HumanHap550 BeadChip, which depending on different factors could affect the results. After comparing 551642 SNPs in the test samples (with extra criteria such as age of MS onset, severity etc), a total of 242 susceptibility SNPs were identified. Of these, 65 were found within the MHC locus on chromosome 6 (the HLA locus). This confirms what older studies have found but this study also found a variety of non-MHC related genes which also show high frequency in MS patients, some of which are the glypican proteoglycan 5 found in plasma membranes and is implicated in brain patterning, synaptic formation, axon regeneration and are found in MS plaques (Baranzini. S et al. 2009, Filmus. J et al 2008) and Parkin which is a component of the E3 ubiquitin ligase complex used in protein degradation and has been shown to have a role in the development of juvenile-onset Parkinson's disease. It has also been known to affect mitochondria and apoptosis of neurons, which are important in pathogenic MS (Rankin. C et al 2011, Baranzini. S et al. 2009).

In the same year another association analysis was performed in Australia and New Zealand (Bahlo. M et al. 2009). This study used 1618 MS test samples and used share data of 3413 for controls. The study was also further independently replicated with 2256 test samples and 2310 controls. The samples obtained were from patients with European descent and the control samples were from the United Kingdom and the US (with European descent). In this study again another Microarray chip was used (Illumnia© Infinium Hap370CNV). The results obtained showed high association with the MHC/HLA locus (strongest association in this locus was the HLA-DR15 gene), as well as other previously found sites. Novel sites found include a region on chromosome 12 which contains 17 known genes and another locus on chromosome 20 which contains a gene for a member of the tumour necrosis factor receptor superfamily and has been known to be associated with rheumatoid arthritis and Graves's Disease. It is expressed on B cells, dendritic cells, macrophages and microglia, and it is known to be a regulator in immunity.

As can been seen with the above studies, as technology has become more advanced, our ability to detect more associations between SNPs allow the identification of more susceptible genes associated with MS and gives a better chance at treating the disease, instead of just the symptoms.

Amiloride Sensitive Cation Channel 1

It was through a genome wide association study that the gene ANCC1 (Amiloride-sensitive Cation channel Neural 1) became a potential susceptible gene for MS (Bernardinelli. L et al. 2007). This study aimed to avoid heterogeneous samples, as this may have an effect on statistical efforts to determine moderate associations, and so the focus for the study was put on the population of Nuoro Sardina in Italy, which has been genetically isolated and also has a much higher rate of MS in the population, than the Italian mainland. To begin the study a sample was taken from people with MS and their family members (how the samples were obtained is not stated) and a small number of microsatellites were tested from these samples in the previously found chromosome 17 region for MS association. This showed one significant association, the microsatellite D17S798. The study was further investigated by performing a bioinformatic screen of the DNA region surrounding D17S798 and extending out to the next two microsatellites either side of it. The results showed that the ANCC1 gene is a potential susceptibility gene. The exons and 3'untranslated region (3' UTR) of ACCN1 were resequenced. Using these sequences the SNPs in these areas were tested for association to MS and it was found that there was a significant association between MS and the SNP rs28936 located in the 3' UTR of ACCN1. There had already

been evidence from previous experiments which suggest that ACCN1 might play a role in MS pathogenesis. Also, the results from this study gave an increased reason to investigate the gene further.

The gene ACCN1 (Amiloride-sensitive Cation channel Neuronal 1) codes for the acid sensing ion channel 2 (ASIC2/MDRG) (Wemmie. J et al. 2006, Xlong. Z et al. 2008, Askwith. C et al. 2004, Xhang. G et al 2009). It belongs to a family of ASIC proteins all encoded by the ACCN genes, ACCN2 for ASIC1 (which has two splice variants a&b) and ACCN3 for ASIC3 and ACCN4 for ACIS4 which both do not show any splicing variants. This channel is mainly permeable to sodium but is also known to allow other cations such as calcium, potassium, hydrogen ions and lithium to pass (Wemmie. J et al. 2006, Xlong. Z et al. 2008, Askwith. C et al. 2004). It has two splice variants ASIC2a (MDRG1) and ASIC2b (MDRG2). Of the two subunits, only ASIC2a can support proton gated currents. AISC2b can only couple with other subunits to modify their properties (Wemmie. J et al. 2006). ASIC2/MDRG is a member of the larger degenerin epithelial sodium channel superfamily and is predominately found in cells originating from the dorsal root ganglia, which include sensory neurons, the CNS and the heart (Bassilana. F et al. 1997, Lingueglia. E et al. 1997, Villa-Carriles. W et al. 2006). ASIC2 is thought to play a part in touch sensation along with ASIC3 (Price. M et al. 2000) and it has been suggested that it is found in the post synaptic membrane of granule and purkinje cells in the cerebellum. The two subunits of ASIC2 combine with each other and other members of the ASIC family to form multimeric subunit channels which are activated by extracellular acid/proton accumulation (decrease in pH). Different characteristics are observed from different combinations of the subunits and they are found in various areas of the CNS and PNS (Lingueglia. E et al. 1997, Feldman. D et al 2008). The structure of the ASIC2 channel was shown to have two intracellular amino and carboxyl ends and a large extracellular loop which is thought to contain the pH sensitivity for channel activation (Figure 2) (Saugstad. J et al. 2004). A channel which is equivalent to the mammalian ASIC2 was found to induce neurodegeneration in *Caenorhabditis elegans* (*C. elegans*) when mutated in the presence of other degenerins (DEG-1, MEC-4 and MEC-10) (Bassilana. F et al. 1997).

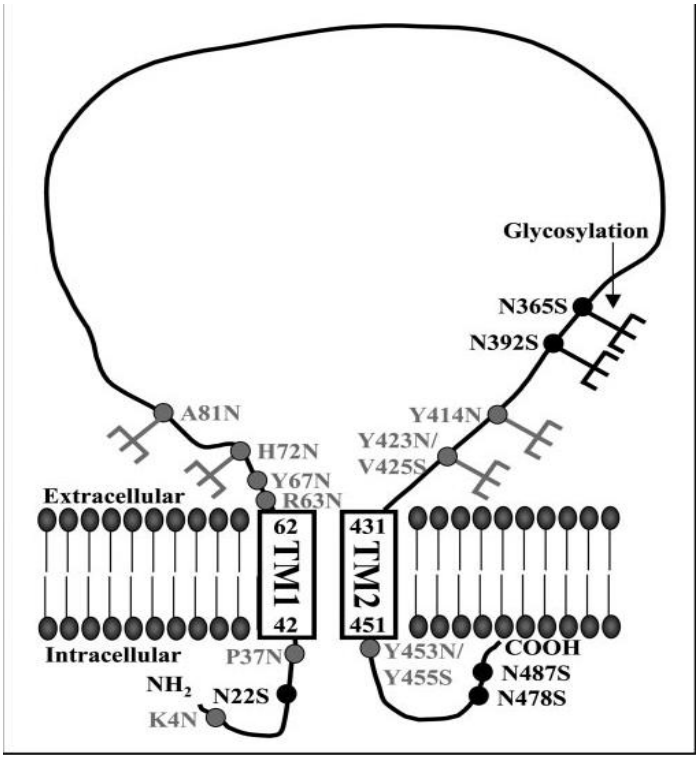


Figure 2: A representation of ASIC2a topography based on glycosylation and antibody permeabilization studies. Diagram shows amino and carboxyl termini are intracellular, two transmembrane domains and a large extracellular loop domain (Saugstad. J et al 2004).

Much research has gone into the expression of ASICs. It was found in 2007 that ASIC1 may contribute to axonal degradation in autoimmune inflammation (Friese. M et al. 2007). Firstly, it was known that the ASIC channels are activated with a drop in extracellular pH, so it was tested to see whether there was a lower pH in CNS tissues than in control tissue, which would contribute to over expression of the ASIC1 channel. It was found in mice with experimental autoimmune encephalomyelitis (EAE) (an induced form of MS in mice) that there was a lower pH in these tissues, which favours the longer activation of the ASIC1. It was then shown in mice with knocked down ACCN1 gene that lack of the ASIC1 protein actually reduced axonal degradation. It was also found that inhibition of the ASIC1 protein (using Amiloride, an antagonistic drug used to treat hypertension and congestive heart failure) reduced axonal degeneration (Figure 3) and more specifically Plasmotoxin-1 (which directly targets the ASIC1a subunit) was also shown to directly reduce axonal degradation. The information found in this study suggests that treatment with Amiloride or other drugs which block ASIC1 receptors could decrease axonal degradation in MS patients. This study highly suggests that ASICs are involved in MS pathology, and gives further evidence to investigate ASIC2 more closely.

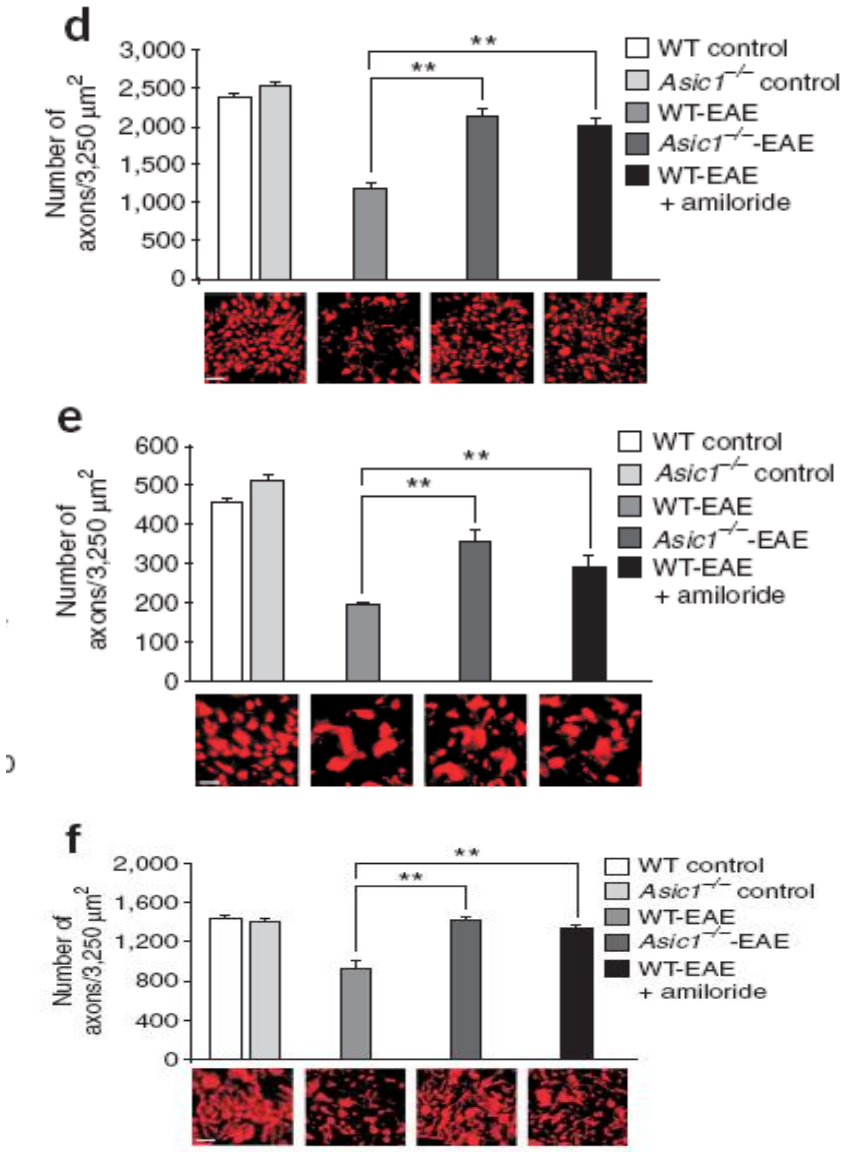


Figure 3: Evidence of axonal protection in EAE mice via Amiloride treatment in the d) corticospinal tract e) dorsal column and f) optic nerve. All graphs show wildtype and Asic1$^{-/-}$ controls, Wildtype with EAE, Asic1$^{-/-}$ with EAE and finally wildtype with EAE and Amiloride treatment. When comparing the number of axons in a 3,250µm^2 area there was significant axons found in the Asic1$^{-/-}$ with EAE mice (as was found in a previous experiment in the study) and in the wildtype with EAE and Amiloride treatment mice, suggesting that Amiloride treatment mimics the effect of the Asic1$^{-/-}$ knock down. This result suggests that down regulating ASIC1 in either EAE or MS may lead to recovery of axons, and suggests a good therapeutic drug, Amiloride (Friese. M et al 2007).

Research into the ASIC2 specifically showed that the subunit ASIC2a modulates the activation of proton currents for the ASIC1 unit in the hippocampus (Askwith. C et al. 2004). Study of the subunits showed that current was provided by an ASIC1a/ASIC2a heterodimer, with the ASIC1a subunit providing current amplitude and ASIC2a providing desensitisation and the response to modulatory agents. This study also suggests that the ASIC2 channel is involved in neurological

function, which with the evidence from the genome wide study, gives more reason to investigate the ACCN1 gene further for its potential as a MS susceptibility gene.

MicroRNA activity and pathways

As the mutations were found in the 3'UTR of the ACCN1 gene, it was suggested that the SNPs might be causing a disrupted microRNA binding. microRNAs (miRNAs) are small RNA molecules (generally around 22 nucleotides long) that regulate the expression of gene transcripts via binding to the messenger RNA (mRNA) and preventing translation to protein (post transcriptional regulation) (Zhang. X et al. 2010, Carthew. R et al. 2009, Miyoshi. K et al. 2010). miRNAs are often transcribed in clusters in the genome at various chromosomal positions. A cluster commonly has miRNAs which target the same cellular pathways. One miRNA can also target many mRNAs due to imperfect complementarily between miRNA and its targets (Zhang. X et al. 2010). They are similar to small interfering RNAs (siRNAs) which are involved in the cell's reaction to viral infections known as RNA interference or RNAi, but they are processed differently. siRNAs are produced from long double stranded RNAs while miRNAs are produced from a single stranded transcript that fold into a hairpin (Carthew. R et al. 2009). Hundreds of miRNAs have been found in a large group of animals and plants, which suggests that miRNAs are common and highly abundant as a control mechanism for protein expression.

In the canonical miRNA biogenesis, miRNAs are encoded in the genome either as single transcripts or in clusters, and are transcribed by RNA polymerase II as a long transcript known as the pri-miRNA. The transcripts are both 5' capped and 3' polyadenylated like an mRNA transcript. The pri-miRNA is single stranded and once transcribed forms one or several hairpin loops. Hairpin loops are single stranded RNA which form complementary base pairing with itself, forming a stem and loop structure. This structure is trimmed while it is still in the cell nucleus at the 5' and 3' ends by an RNase III family member named Drosha which is able to cleave double stranded RNA. Drosha is part of a protein complex known as the Microprocessor complex. The Microprocessor complex allows for accurate cleavage while the Drosha component physically cuts the transcript generating pre-miRNAs that are 70-90 nt long molecules. In animals the pre-miRNA is moved out of the nucleus into the cytoplasm via the Exportin 5 protein. In the cytoplasm Dicer (another RNase III which is also capable of cleaving double stranded RNA) (Merritt. W et al. 2010) cleaves the loop of the hairpin and liberates the short approximately 22 nucleotides long double stranded RNAs. The stem can have more than one miRNA and they commonly control genes involved in the same system. In plants this reaction takes place in the nucleus and the short double stranded RNA is moved into the cytoplasm. The double stranded miRNA is then split as it becomes associated with

the RNA induced silencing complex (RISC) where the main component is an Argonaute (Ago) protein (Carthew. R et al. 2009, Filipowicz. W et al. 2008, Winter. J et al 2009). The Argonaute proteins are a family of endonucleases which cleave sequences that have complementarity with the small RNA that is bound (the bound miRNA guides the RISC). The Ago protein is expressed in mammals and is used in the processing of miRNAs. They have four domains, PAZ (shared with Dicer), PIWI, N and Mid domains. The PAZ domain has RNA 3' terminal binding activity which guides the binding of the strand. The 5' of the RNAs strand interacts with the Mid domain. The RNA strand is bound to the protein via interactions with the sugar phosphate backbone, allowing the exposure of the bases for binding to the target sequence. Bases 2-6 on the miRNA must be exposed as they make the seed sequence which directly binds to the target. The PIWI domain is capable of cleaving the target sequence to bring about silencing, however not all Ago proteins have this ability. Eight Ago proteins have been found in Humans, where four are able to associate with miRNAs and siRNAs. It is yet unclear what the specific functions of all of the Ago proteins are and why some are capable of cleaving and others are not. Ago becomes associated with other protein subunits to produce the RISC which allows for an accurate binding to the mRNA strand and therefore an accurate prevention of translation (Iwasaki et al 2009). The strand which is associated with Ago is chosen for its thermodynamic stability at the 5' end, where the strand is less stably based paired with the other strand. The left over strand is known as the miRNA* strand and is usually degraded. The bound mature miRNA in the RISC locates the appropriate sequence on the mRNA strand and depending on the degree of base pairing there will be a response. If there is a high level of base pairing between the miRNA and the mRNA then the mRNA is cleaved by Ago. If there is a lower level of base pairing between the miRNA and the miRNA then the mRNA is removed from the translation machinery and placed in an area of the cell known as the P-body for storage. The mRNA could later be moved back to the translation machinery or degraded. Figure 4 below illustrates the miRNA biogenesis pathway.

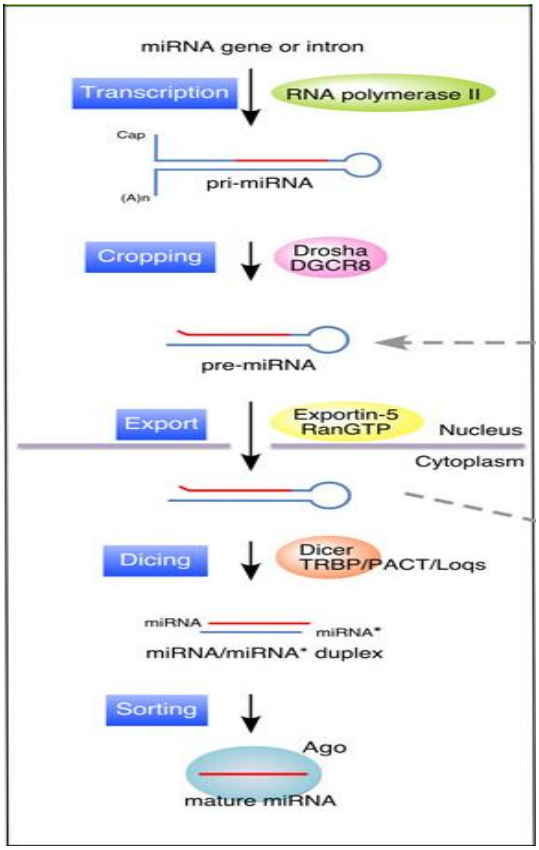


Figure 4: Figure represents the miRNA biogenesis in animals as described in the text. (Miyoshi. K et al. 2010).

The Microprocessor unit is not always necessary for cleavage, there are further non-canonical pathways which have been found to produce working miRNAs (Miyoshi. K et al. 2010, Winter et al. 2009). Pre-miRNA transcripts can be released from introns in their full pre-miRNA structure, and enter the normal miRNA processing pathway are known as Mirtrons. miRNAs can also be generated from small Nucleolar RNAs. Small Nucleolar RNAs (snoRNAs) are RNAs which reside in the nucleus and are part of small nucleolar ribonucleoprotein (snoRNP) complexes. The snoRNPs modify ribosomal RNAs (rRNA) to give them their enzymatic abilities. Some snoRNAs form two hairpin structures, which are connected by a hinge that can be processed by Dicer into miRNAs. This process does not require Drosha or the Microprocessor complex. The RNAs are functional both as snoRNAs and miRNAs. This finding suggests that there is an ancient link between snoRNAs and miRNAs. miRNAs can also be generated from endogenous short hairpin RNAs (endo-shRNAS) which are Dicer dependent. From these endo-shRNAs, two hairpin loops connected by a hinge exist in a pre-miRNA state. From this structure only the second hairpin loop produces any miRNAs. It is thought that the shRNAs are processed by endonucleases other than Drosha, and then finally processed by Dicer. Studies have shown a shRNA locus in the isoleucine tRNA gene, which primarily forms the clover leaf structure of a tRNA, but also has a secondary transcript which contains miRNA precursors. And finally the miRNA miR-451 has been found to exist in the loop structure of the its pre-miR-451, and that production of the mature miRNA does not require Dicer

as evidenced by high levels of miR-451 in Dicer null mice but low levels in Drosha null mice. This suggests that Drosha processes miR-451.

Examples of MicroRNA expression involved with Multiple Sclerosis

miRNAs have been found to play a role in many processes across the spectrum of cells in the body including brain development and degeneration (Zhang. X et al. 2010, Carthew. R et al. 2009, Miyoshi. K et al. 2010, Bredy. T et al. 2011, Kloosterman. W et al. 2006, Friedman. J et al. 2009). This has been prevalent in studies on MS, where various miRNAs have been found to have an effect on MS pathogenesis. The following are some examples of studies involving miRNAs which have recently been linked to MS in the past two years.

In 2009 (Otaegui. D et al. 2009), a group studied the expression difference of miRNAs in patients with MS. The group used samples of PBMC (peripheral blood mononuclear cells, blood cells which contain a round nucleus such as those involved in the immune system) and looked at the expression patterns of 364 miRNAs, in relapse (four samples), remitting (nine samples) and control (eight samples). After analysis two miRNAs were shown in the relapse stage of the disease to be up regulated, hsa-miR18b and hsa-miR599. The analysis results of these miRNA suggest that both are involved in the mechanism of the relapse period. The analysis also showed the miRNA hsa-miR96 is up regulated in the remission stage. When looking for the target genes of hsa-miR96, it was found that the genes are involved in immunological pathways such as interleukin signalling and also in the Wnt signalling pathway. For the other two miRNAs found, there was no significant involvement of the target genes in specific pathways, but it has been suggested that the up regulation could be utilised as a biomarker for fast identification of relapse stages in MS patients. This study, while small, showed the importance of miRNA expression patterns when looking at the mechanisms which may contribute to MS pathogenesis.

Later that same year another paper was published which looked at MS lesions and their miRNA expression patterns (Junker. A et al. 2009). This study looked at the miRNA expression profiles from both active and inactive lesions. Twenty samples were used and nine control samples, which were obtained from preserved samples. It was found that astrocyte cells from active lesions contained all ten miRNAs that were found to be activated strongly in active lesions in general, of which included miR155, which is known to be involved in controlling immune responses. It was also found that the expression of cytokines induced the expression of miR155 when tested *in vitro.* The up regulated miRNAs from the active lesions were then identified with their target mRNA, and it was found that three of the miRNAs discovered (miR34a, miR155 and miR326) all

targeted the 3' UTR of CD47 (miR155 bound at two sites), which inhibits the phagocytic activity in macrophages and also inhibits cytokine production in dendritic cells (Timo. K 2010, Matozaki. T et al. 2009). CD47 is considered as a 'self' signal to avoid phagocytosis of healthy cells. Up regulation of miRNAs which target CD47 will lead to down-regulation of the mRNA transcript and so less protein, which will stop inhibition of macrophages and is thought to lead to the phagocytosis of myelin. CD47 also stop the production of cytokines in dendritic cells (Ni. Choileain et al. 2011, Junker. A et al. 2009).

Later the same month, another paper was published which looked for the expression profiles of miRNAs between relapse-remitting patients and healthy controls in order to differentiate between disease and healthy states (Keller. A et al. 2009). Twenty relapse-remitting patients were sampled and 19 controls were profiled, and 165 miRNAs were found to be either up- or down-regulated in MS patients compared to control samples. The strongest signal was given by hsa-miR145, allowed at specificity 89%, sensitivity 90% and accuracy of 89.7%. This was a distinct identification of MS in patient samples, allowing for a strong indicator of MS over healthy samples and so a strong candidate for a biomarker. Further investigation into the miRNAs, showed that 43 miRNAs have already been found to be mis-regulated in other human diseases. The final 122 miRNAs were at this time exclusive to MS. This study has shown two major advantages in studying miRNAs in MS. Firstly, this study has shown that miRNAs can be used as biomarkers to indicate MS for diagnosis. And secondly, it has also shown that there is a vast amount of mis-regulated miRNAs which could all be implicated in the pathogenesis of MS, giving a place to start looking for treatment options in strongly mis-regulated miRNA/genes or in strongly affected cell pathways.

In June 2010, a paper was published looking at the miRNA expression in T regulatory cells in MS (Santis. G et al. 2010). Twelve relapse and remitting patients and fourteen controls were sampled. T regulatory cells were extracted and tested for miRNA dysfunction between relapse, remitting and controls. Twenty-three miRNAs were differentially expressed between MS patients and healthy controls. Members of the miR106b-25 miRNA family (miR106b, miR93 and miR25) were found to be up-regulated in MS patients along with two miRNAs from the miR17-92 paralog cluster (miR19a and miR19b), among others which were not statistically significant. miR106b and miR25 have been known to be important in the TGFβ signalling pathway, for control of the cell cycle inhibitor CDKN1A and for control of the pro-apoptotic gene BLC2L11. This finding suggests that as TGFβ is so important in T cell regulation and maintaining tolerance of 'self' in cells which undergo phagocytosis, blocking of the signalling pathway could suppress Foxp3 (a T regulatory cell marker) and so lead to altered T regulatory cell function (Santis. G et al. 2010). Furthermore, miR210 was also found in this study which targets Foxp3 and was up regulated in T regulatory cells. This may also contribute to the altered cell function in MS patients. Finally, when comparing

the levels of T regulatory cells against the levels of T effector cells, it was shown that there is an increase in miRNA levels in T regulatory cells but not in T effector cells in MS patients compared to healthy samples. This study gave strong evidence for miRNA mis-regulation causing a direct effect on the function of T cells known to have altered function in MS and gave a basis for further research into the theory suggested over the suppression of the TFGβ pathway. It also suggests putative miRNAs to target for therapeutic purposes.

Later the same year a further paper was published focusing on miR17 and miR20a and their ability to inhibit T cell activation genes and their subsequent down-regulation in MS patients (Cox. M et al. 2010). Fifty-nine MS patient samples (with a mixture of all the different MS subtypes) and 37 controls were tested for up or down-regulation of miRNAs using 733 known miRNAs. Of the 26 down-regulated in the MS samples, miR17 and miR20a were shown to be statistically significant and so became the main focus of the study. Both miRNAs have been shown to be involved in immune function being found together in the same miRNA cluster (miR17-92). These miRNAs control the translation pathway for lymphocyte differentiation and target the pro apoptotic signal Bim (Belver. L et al. 2011). Over-expression of miR17 has been found in B cell lymphoma and other cancers and as such is suggested to maintain undifferentiated cells and resistance to apoptosis (Cox. M et al 2010, Belver. L et al. 2011). This study showed by using knock in and knock down experiments, that both miRNAs have a role in the control of T cell activation genes. On top of the down regulation of the miRNAs, MS patient whole blood mRNA extraction showed an up regulation of T cell activation genes, complementing the miRNA findings. This strongly suggests a role for miRNAs in T cell disfunction in MS and suggests a potential therapeutic target.

Lastly in December 2010 a study was published which looked at the altered expression of miR17-5p in CD4+ lymphocyte (T helper cells) cells in MS patients (Lindberg. R et al. 2010). 365 miRNAs were analysed in lymphocyte cells in relapse and remitting MS patients. It was shown that miR15-5p was up regulated in CD4+ cells from MS patients. The miRNA cluster miR17-92 is known to contribute to the development of autoimmunity and a lymphoproliferative disease in mice (Thamilarasan. M et. al 2011, Belver. L et al. 2011). No other members of this cluster were found to be mis-regulated in the CD4+ cells however there was some change in miRNA expression from this cluster in B cell lymphomas, which has been seen previously as disruption of this cluster can lead to B cell lymphomas. In this case the down-regulation of miR-92 was found in the B cell lymphoma (Cox. M et al. 2010, Belver. L et al. 2011). miR15-5p (which was found to be up regulated in CD4+ cells) was found to target the PI3K/Akt pathway. It was shown that members of this pathway were shown to have altered expression, in particular PI3KR1 and PTEN. As PI3K is essential for lymphocyte development, activation and survival, this strongly suggests a link between miR15-5 and T cell disfunction. Also, knocking down miR17 supported the link between

altered miRNA expression and MS as it was shown that the knock down altered target protein expression. Furthermore, it was found that stimulating the cells lead to mis-regulation of other miRNAs such as miR193a which was strongly up-regulated and miR497, miR1 and miR126, which were down-regulated. This study gives more information on the miR17-92 cluster and shows that it is highly likely to be a susceptible area for MS patients. Also, as miR17-5p was so prominent it suggests that this miRNA may be a potential therapeutic target.

With the information found in the Bernardinelli. L et al. study, the aim of this study was to investigate the SNP in the 3' UTR of ACCN1, to determine whether there is a connection to miRNA expression in the 3' UTR and if so to understand the mechanism by which mis-expression has taken place. Therefore, it is hypothesised that firstly, ACCN1 expression is under the control of an unknown miRNA. And that secondly, an SNP present in the 3'UTR of the ACCN1 gene is causing a disruption to the regular binding of the unknown miRNA, leading to over expression of Acid Sensing Ion channel 2.

Construct Cloning

Modified Promega pGL3 plasmids with the multiple cloning site transferred to the 3' end of the Luciferase gene were cut with SacI and NheI restriction enzymes. The 3'UTR region of ACCN1 gene was amplified by PCR using two plasmids called AA and GG obtained from the Bernardinelli research group. These plasmids contained two forms of the ACCN1 3'UTR. During the PCR SacI and NheI restriction sites were added to the end of the fragments and after isolating the PCR products from 1% agarose gel, these were also digested with the two restriction enzymes. The cleaved plasmids and samples were ligated together to incorporate the 3' UTR's into the plasmid at the 3' end of the luciferase gene. This allows the 3' UTR alleles AA and GG to act as the miRNA binding site for the luciferase gene in the plasmid.

Site directed mutagenesis

The AA plasmid obtained from the Bernardinelli research group was further modified to create the mutations found in the sequence data. The 3' UTR in the AA plasmid was amplified via PCR using mutant primers with the SNP mutations at the appropriate locations (Primers listed in 5' direction for both the forward and reverse reactions:

Orientation	Forward	Reverse
Mutant1	CTGCATGCTCTGACTTCTTGTGGCGCCACCTCCACGT	ACGTGGAGGTGGCGCCACAAGAAGTCAGAGCATGCAG
Mutant2	CTGCATGCTCAGACTTCTTATGGCGCCACCTCCACGT	ACGTGGAGGTGGCGCCATAAGAAGTCTGAGCATGCAG
Mutant1-2	CTGCATGCTCTGACTTCTTATGGCGCCACCTCCACGT	ACGTGGAGGTGGCGCCATAAGAAGTGGGCAGCTTGG
Mutant3	CCAAGCTGCCCACGGGGCTTCGGCGCCAAAGGTGA	TCACCTTTGGCGCCGAAGCCCCGTCACAGGCACTGG
Mutant4	CCAGTGCCTGTGACGGCGGCACAGCAGCCAGCGGGT	ACCCGCTGGCTGCTGTGCCGCCGTCACAGGCACTGG

Table 1: Showing the primers for the forward and reverse reactions.

The primers overlap in the middle of the sequence to allow replication to take place. As replication happens, the mutation is incorporated into the fragment. The mutant primers also incorporated SacI and NheI sites at the ends of the 3' UTR to allow the insertion of the new mutant fragment back into the pGL3 plasmid. This was performed via a digestion with SacI and

NheI restriction enzymes on the fragments and the pGL3 plasmid, followed by ligation into the plasmid.

Cell Plating and Transfection

MCF7 cell cultures were split and grown to a 45-50% confluence in 100µl growth media (Invitrogen DMEM 10938 with 10% FBS and 1% glutamine). Cells were counted, diluted and transferred into 96 well plates and allowed to rest and adhere to the plate overnight. Media was removed and replaced with 100µl Optimem (Invitrogen) to wash the cells, then 50µl of Optimem was added and then removed just before the transfection agents were added. Lipofectamine (Invitrogen) was diluted in Optimem at 0.5µl Lipofectamine to 100µl Optimem. The constructs/siRNAs were diluted in Optimem at 100µl Optimem to the specific amount needed to make all of the constructs/siRNAs to a concentration of 20µm in 100µl. These varied due to the different concentrations of all of the stock solutions of the constructs. The siRNAs stock solutions were all the same concentration and were all diluted and used at 0.6µl per 100µl Optimem. 100µl of the Lipofectamine/Optimem mix was then added to 100µl of the construct/siRNA mix. The construct/siRNA mixes were done with three variations, one contained just the construct in 100µl of Optimem, another with construct and the Dicer knock down siRNA in 100µl of Optimem and the last with the construct and the Scrambled negative control siRNA in 100µl of Optimem. This gave an overall volume of 200µl of the Lipofectamine/construct/siRNA/Optimem mixture. 50µl of the mixture was then added to each of the wells (enough for four repeats of one construct). The cells were left for 5 hours at 37˚c, then the mix was removed and changed for growth media.

Luciferase Assay

100µl growth media was removed from the wells and replaced with 100µl of cooled PBS (phosphate buffered saline), the plate was shaken for one minute, the PBS was removed with an aspirator and the plate was put on ice. 75µl of lysis buffer was added to the wells and the cells were scratched off the bottom of the wells in a forward/backwards motion using the pipette tip for ten seconds. The plate was then spun for two minutes at 2000 RPM. 37.5µl of the lysate was removed from the plate and transferred to a Luciferase plate in the same order. 25µl of Luciferase

substrate was added to the wells and the plate placed into the Luminometer (Victor Light Luminometer).

BCA protein assay

20µl of lysate was removed into a clean plate and 200µl of working reagent was added to each well. The plate was incubated at 37˚c for 30/40min if necessary until the solution turned purple. The samples were allowed to reach room temperature, and then the plate was placed into the Luminometer to record intensity of the colour change.

Cell Culture Maintenance

Cells were maintained in DMEM growth media (Invitrogen DMEM 10938 with 10% FBS, 1% glutamine and 1% antibiotics added). Cells were split using 3ml Trypsin (Invitrogen) to detach the cells from the flask, the Trypsin was neutralised with 4ml of growth media, the cells were counted using a counting microscope slide, diluted with DMEM and split into separate flasks.

Dicer dependency of ACCN1 expression

The Bernardinelli et al. (2007) study showed a clear case to suspect miRNA mis-regulation. The 3' UTR of ACCN1 showed an SNP (in position rs28936) in a genome wide association study for SNPs associated with MS. As miRNAs commonly bind to the 3' UTR of a gene as a form of post transcriptional modification, it is likely that disrupted miRNA binding caused by this SNP, has caused a mis-expression of the ACCN1 gene, which may be a factor in the development/symptoms of MS. To prove this, experiments were devised to test the theory that the miRNA target site has been disrupted for the ACCN1 gene. The experiments are described below. A further experiment was designed to identify the precise miRNA which binds to the 3'UTR of ACCN1 which will be outlined in the discussion; however this has yet to be tested.

Samples were taken at random from a pool of individual samples, one from a group containing one of the SNPs and one from a group containing the other SNP. Using the samples, the 3'UTR of the ACCN1 gene containing the two different alleles rs28936A and rs28936G (known as AA and GG with reference to the base present) were cloned into vectors downstream of the Luciferase gene creating plasmid constructs (please see methods section for the protocol). These vectors were then transfected into MCF7 cells with a siRNA which targets Dicer and knocks down its expression, with a non-specific control siRNA (as a negative control known as Scrambled) or alone. The construct only experiment was performed as a positive control to ensure that the constructs were being taken up and expressed properly. The Dicer test constructs were then compared with the Scrambled negative control to see whether the Dicer knock down had an effect on the expression of Luciferase.

The results indicate that the rs28936A construct showed a higher degree of increase in Luciferase expression when Dicer is knocked down compared to the rs28936G construct (Figure 5). This is shown by the increase in expression of the Dicer knock down over the Scrambled control. This suggests that the rs28936A allele is more strongly controlled by the miRNA pathway than the rs28936G allele. T test analysis showed that there was a significant difference to a percentage of 0.05% for AA but there was no significant difference for GG.

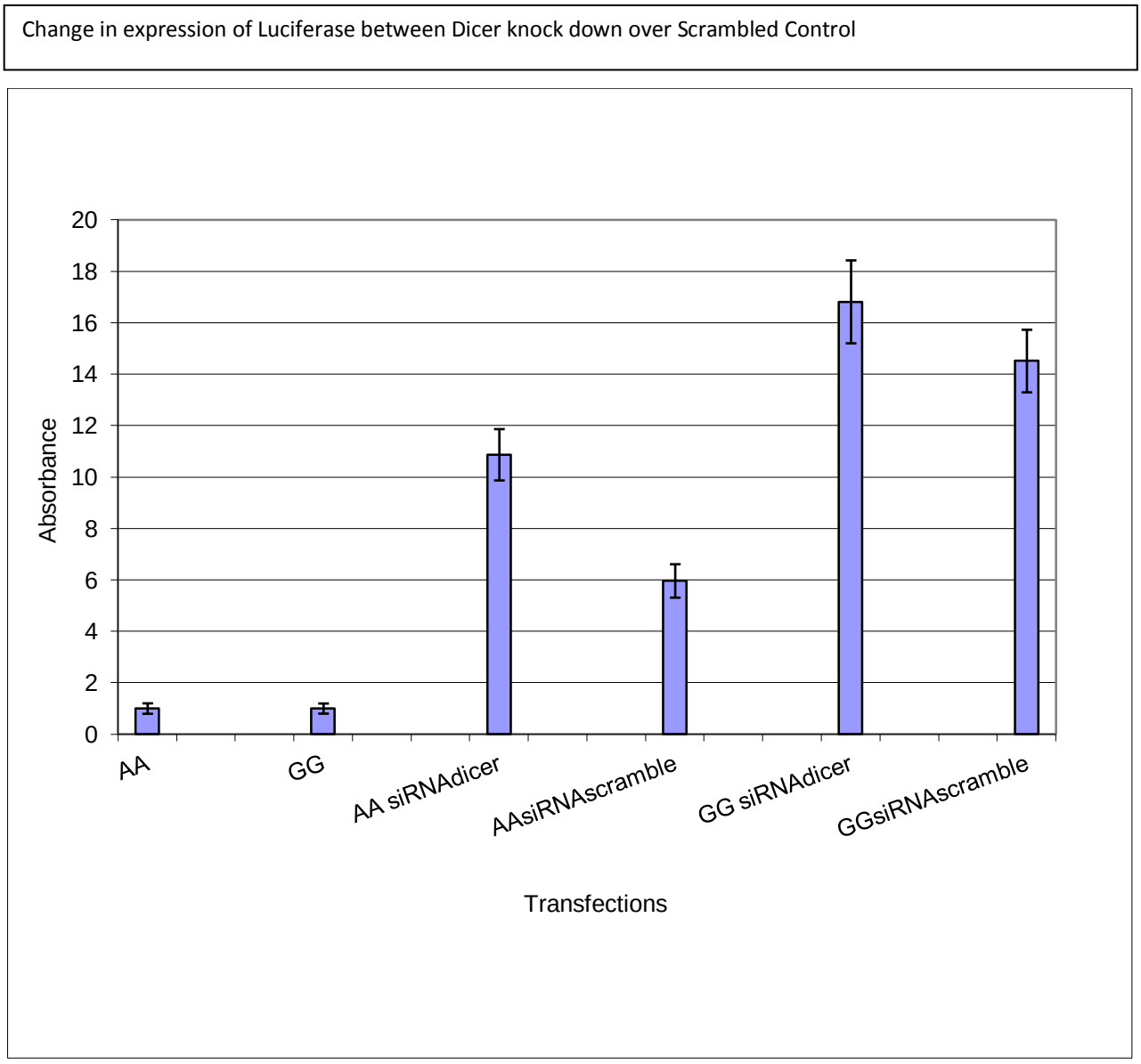


Figure 5: Experiment was performed with four replicates. Values were normalised with respect to the construct only values. This made it easier for statistical analysis and to make it easier to read the graph. AA and GG are positive controls of just the construct. siRNAdicer is the test transfection with the dicer knock down siRNA. siRNAscrambled is the negative control.

To confirm that the only difference between the two constructs is the SNP at the position rs28936, the constructs were sequenced. Surprisingly, the sequencing found a further three SNP mutations in the area surrounding the rs28936 position between the AA and GG constructs (Figure 6). These single nucleotide polymorphisms (SNPs) were at positions rs28639, rs28648 and rs28882.

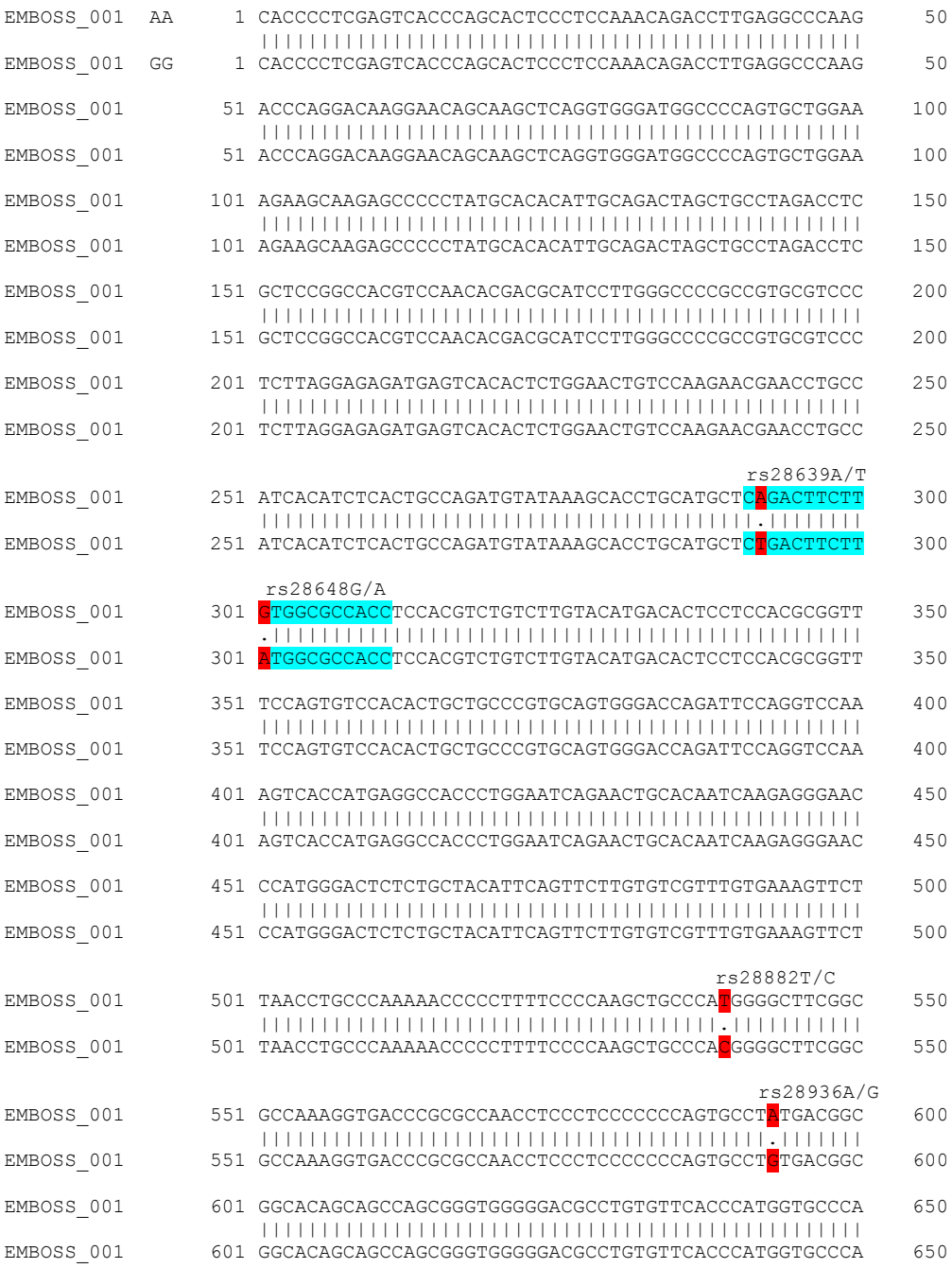


Figure 6: The results of the sequencing alignment. The top line is the AA construct and the bottom is the GG construct. The red shading shows the different SNPs found between the two constructs. These new SNPs were found at positions rs28639, rs28648 and rs28882. The original SNP found is at position rs28936 (the last SNP indicated in red). The blue shading shows a predicted target site for miR652 which includes two of the new SNPs found.

Prediction miRNA target sites in ACCN1 3'UTR

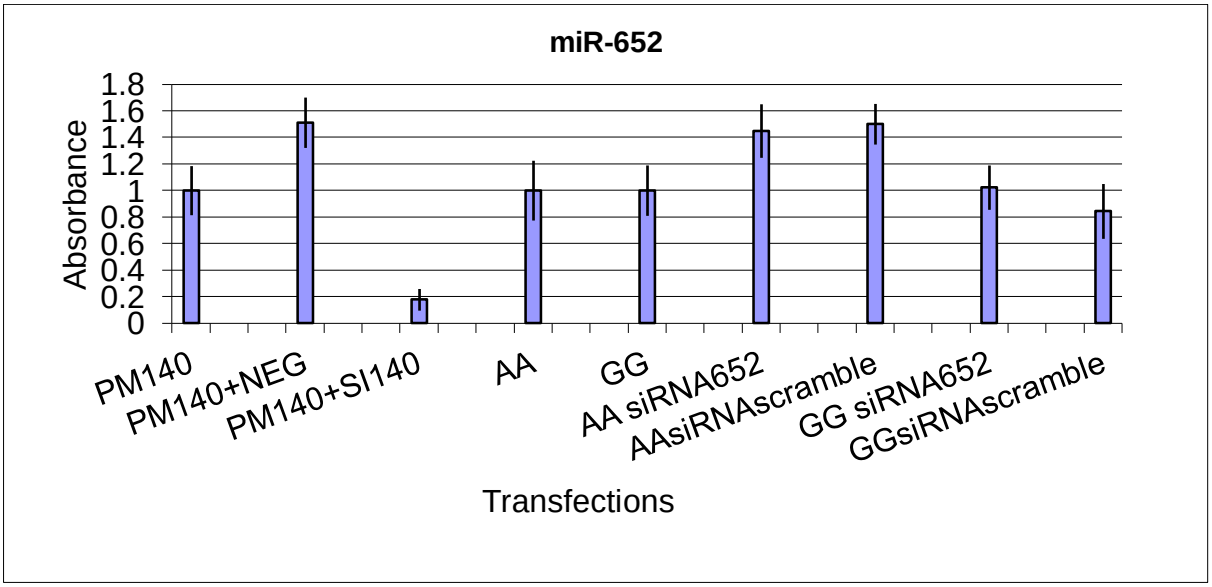
Animal miRNAs have a specific binding style to the target mRNA known as miRNA targeting. The miRNA contains a seed sequence which consists of nucleotides 2-8 at the 5' end of the miRNA. These six nucleotides are crucial for accurate miRNA binding. A site in the mRNA 3'UTR is conserved to complement the seed sequence, in order for accurate and strong binding to happen between the miRNA and the mRNA. It is necessary for complete complementarily binding. If mutations occur in the 3'UTR in the seed sequence, this can strongly disrupt miRNA binding and can lead to an over expression of the mRNA however if there is an Adenosine at the 1 and 9 nucleotide positions this can improve the binding. Another important feature of miRNA binding is a reasonable level of complementary base pairing at the 3' half of the miRNA. It is acceptable for mismatches to occur here, except for at positions 13-16 on the miRNA, which act as a second reference in case there are mutations at the seed sequence. As these rules are the same for all miRNAs, it has made it easier for us to predict mRNA targets once a miRNA is known and databases now allow us to search for mRNAs with matching sequences to the new miRNA.

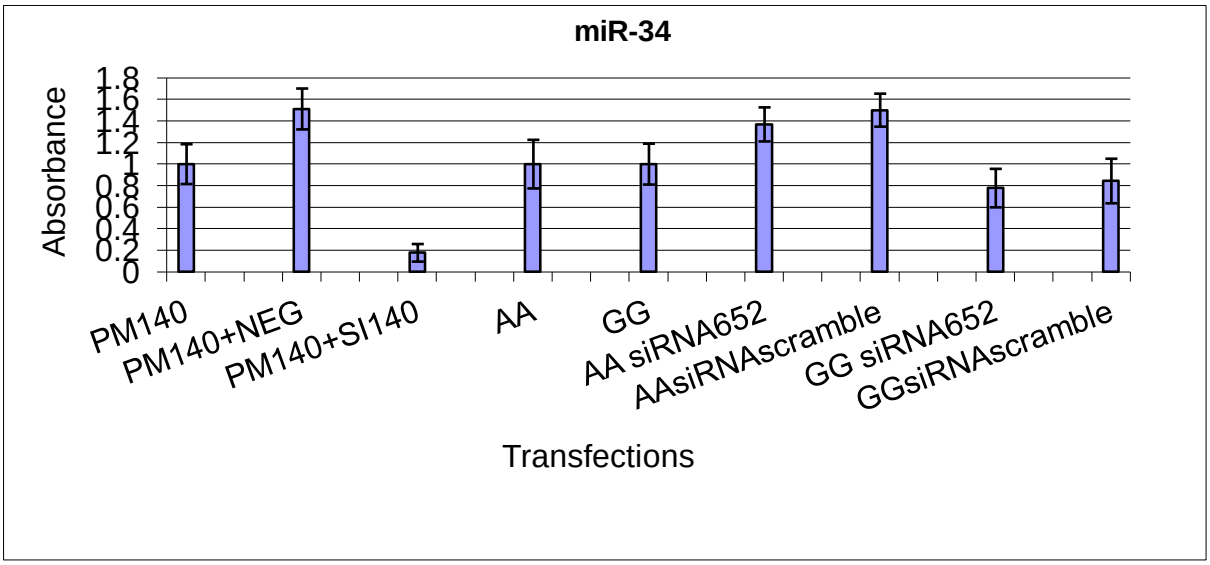
These results raised the possibility that a potential miR652 target site was present in positions 28638-28658.

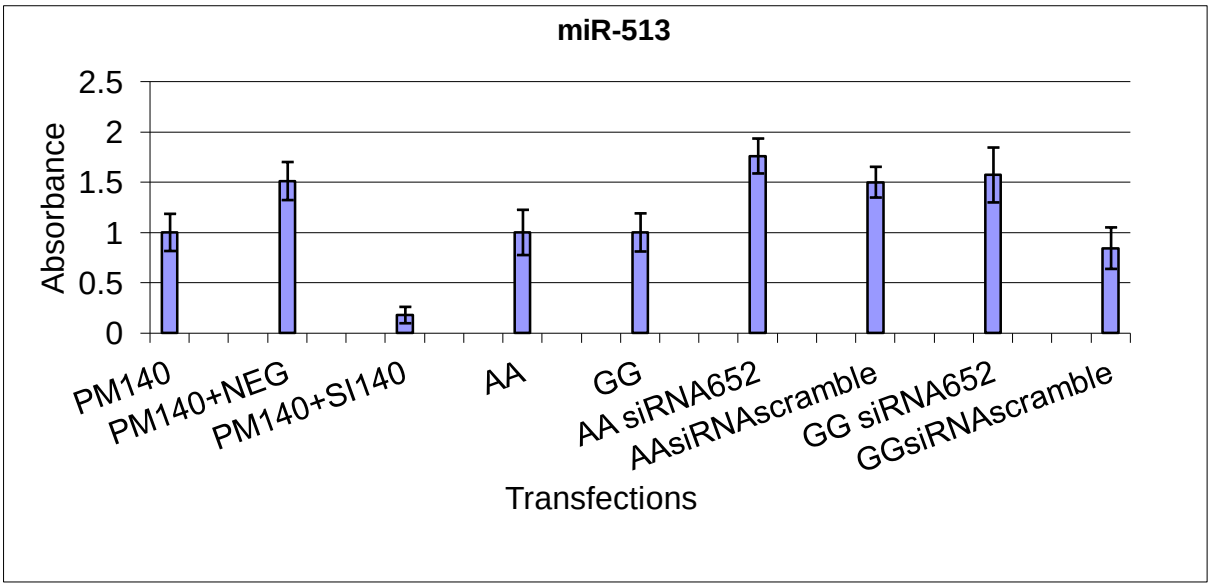
The MiRanada database predicted a variety of potential miRNAs for the original SNP rs28936A/G position. These miRNAs were miR27, miR34, miR220 and miR513. miR652 was also identified as a potential target miRNA for a site which includes two of the new SNPs (rs28639A/T and rs28648G/A) found during the sequencing. As this target site contains two SNPs, miR652 was thought to be a strong candidate miRNA.

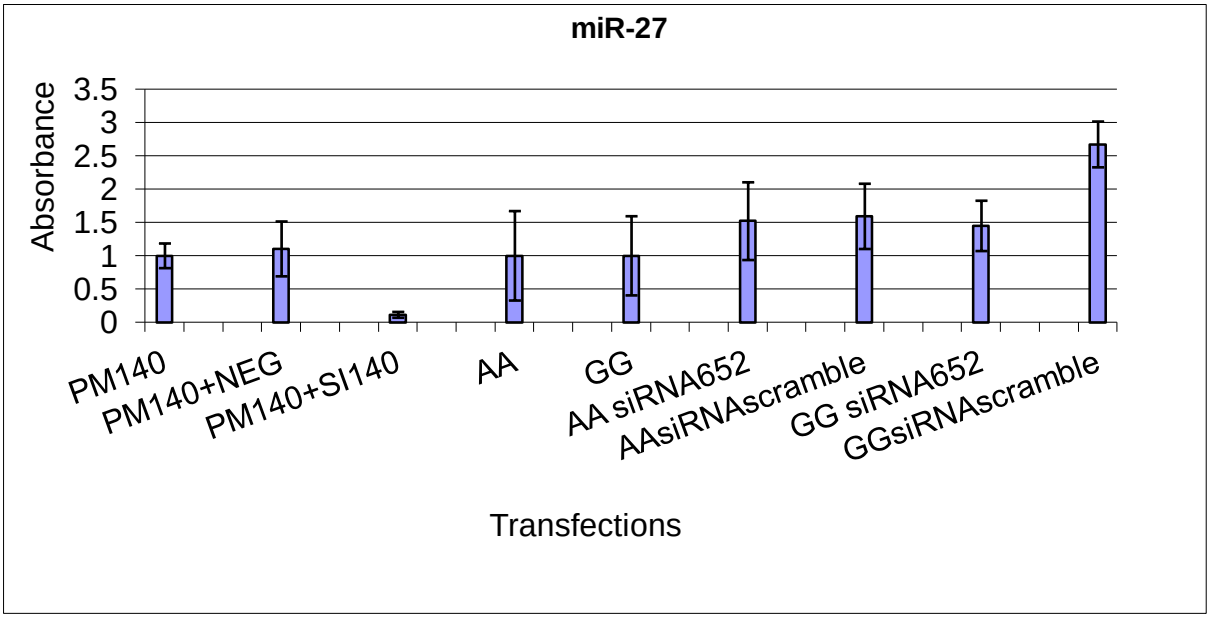
Target validation

To test whether these predicated miRNAs do target the ACCN1 3' UTR, the two previous constructs (which are different in the four nucleotides rs28639A/T, rs28648G/A, rs28882T/C and rs28936A/G) were transfected into MCF7 cells either with or without siRNA mimics of the potential miRNAs in order to determine the difference between the Luciferase expression of each. The graphs generated (Figure 7) show when comparing the level of Luciferase activity with the siRNA mimic and without the miRNA mimic, that none of the predicted miRNAs targeted the ACCN1 3' UTR. This is shown by the level similar expression of Luciferase for both constructs because the differences were not statistically significant.


miR-652
Absorbance
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PM140
PM140+NEG
PM140+SI140
AA
GG
AA siRNA652
AAsiRNAscramble
GG siRNA652
GGsiRNAscramble
Transfections


miR-34
Absorbance
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PM140
PM140+NEG
PM140+SI140
AA
GG
AA siRNA652
AAsiRNAscramble
GG siRNA652
GGsiRNAscramble
Transfections


miR-513
Absorbance
2.5
2
1.5
1
0.5
0
PM140
PM140+NEG
PM140+SI140
AA
GG
AA siRNA652
AAsiRNAscramble
GG siRNA652
GGsiRNAscramble
Transfections



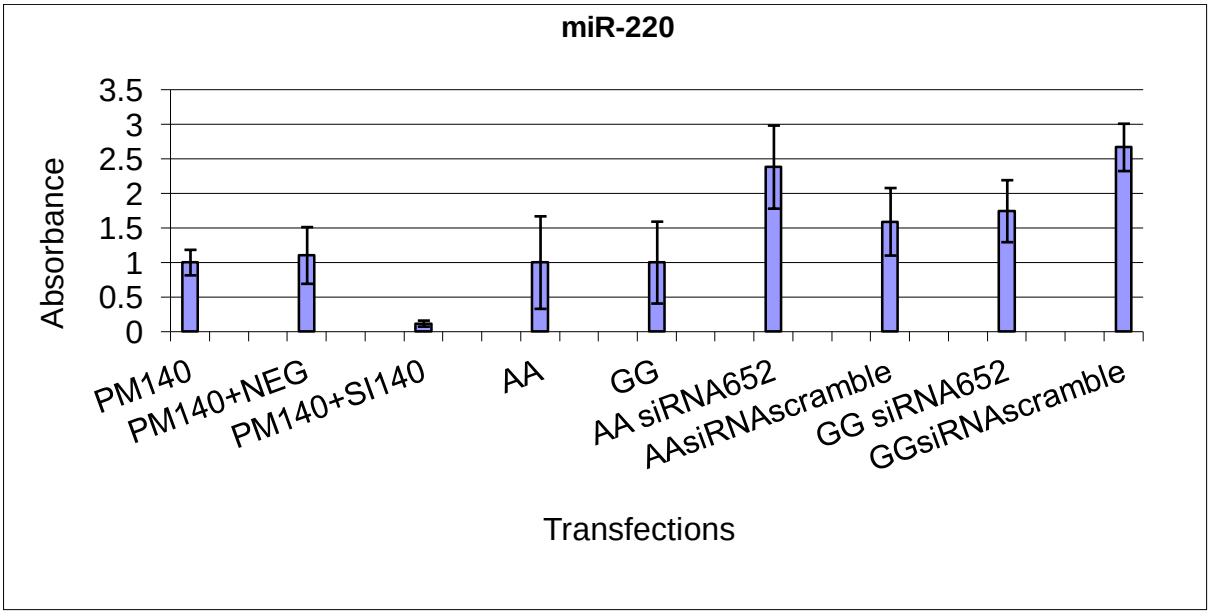


Figure 7: Results of the Target validation analysis. For all graphs PM140 refers to a construct with a Luciferase gene containing a perfect match for miR140 in the 3' UTR. PM140+Negative are an assay of this plasmid with a non-specific Scrambled siRNA and PM140+SI140 are an assay of the plasmid with a siRNA mimic of miR140. These controls were used as verification that the transfection was working properly. The x axis represents absorption of light from the luciferase reaction. The non specific Scrambled siRNA should show no significant decrease in Luciferase activity whereas the siRNA mimic should show a dramatic decrease in activity as the mimic will bind to the binding site and prevent translation. The experiment samples look at the various potential miRNA's ability to suppress Luciferase activity. Here a siRNA mimic of miRNA in question is transfected into the cell along with the constructs AA and GG. If there is a suppression of Luciferase activity this suggests that the miRNA in question is likely to target the 3'UTR of the given form of ACCN1 3'UTR. Of the potential miRNAs tested, none showed any significant decrease in Luciferase activity (Found by T-test analysis). And as such it can be concluded that none of them target the 3'UTR of ACCN1 in either form.

Testing the effect of individual SNP positions on miRNA regulation of ACCN1 3'UTR

As none of the predicted miRNAs showed any targeting of either form of the ACCN1 3'UTR but the two forms were differentially affected by Dicer knock down, we hypothesised that one or more of the new SNPs were located in one or more of the target sites recognised by an unidentified miRNAs. Therefore the roles of the new SNPs found in the sequencing experiment were investigated. The AA construct was mutated so that only one of the SNPs was present in a construct. There were five generated overall. This was done so that the individual effect of the SNP could be established (Figure 8).

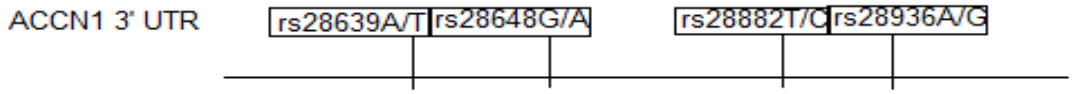


Figure 8: Diagram representing the changes made at the different nucleotide positions, with the letters indicating the nucleotide introduced.

There was also a construct generated where two SNPs, at positions rs28639A/T and rs28648G/A, were present as the potential miR652 was found to contain both SNPs and so it was suspected that both SNPs together might have a strong role in miRNA binding (Figure 6). The new constructs with the individual SNPs were renamed as mutation with its position along the 3'UTR. The naming system is described in Table 2 below.

Construct Name	SNP present
AA	rs28936A
Mut1	rs28639T
Mut1_2	rs28639T/rs28648A
Mut2	rs28648A
Mut3	rs28882C
Mut4/GG	rs28936G

Table 2: Illustrating the new constructs made, their names and the SNPs found in each.

Each construct was transfected into MCF7 cells either alone, with a Dicer knock down or with a non specific Scrambled siRNA as in the original Luciferase expression experiment. As with the first experiment the construct only experiment served as a positive control to determine the accurate

expression of the construct in the cell and to assure the transfection worked. The following graph (Figure 9) shows that there was no significant difference between the expression of Luciferase of the Dicer knock down and the non specific Scrambled control. This suggests that more than one SNP in the 3'UTR of ACCN1 contribute to the reduction in miRNA binding (once more confirmed by T test analysis).

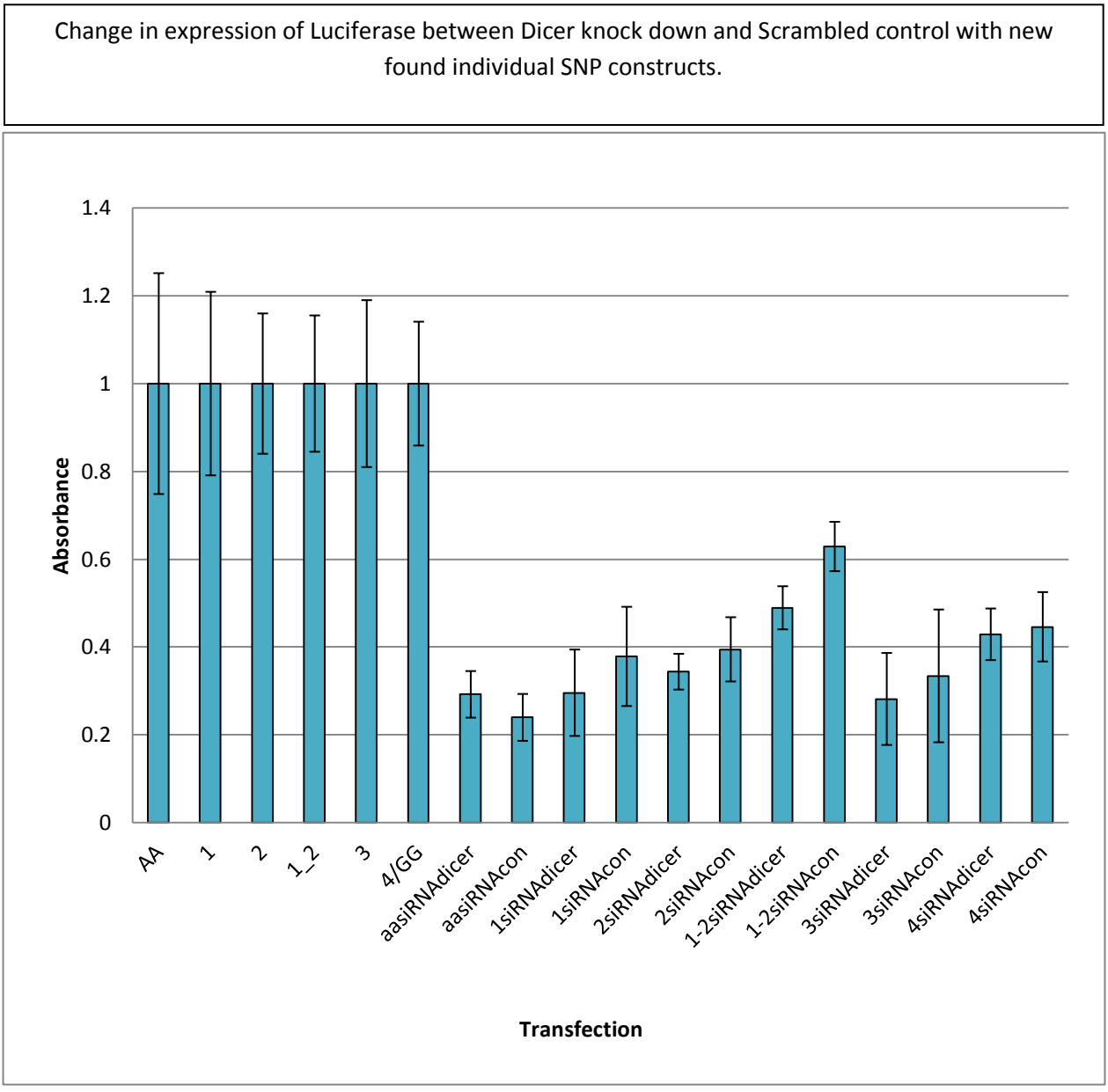


Figure 9: Experiment was performed with twelve replicates. Values were normalised with respect to the construct only values. AA through 4/GG are positive controls. Negative controls are those labelled with Con and the Experiments were all labelled with dicer. The negative controls were compared with the dicer experiments to determine the difference in expression of luciferase.

Genetic studies have been an important tool in discovering the pathogenesis of Multiple Sclerosis. The contributions have suggested a great many genes which each are now thought to play a role in the development of MS, with much evidence to support the involvement of the HLA region of genes (Hafler. D et al. 2007, Oksenberg. JR et al. 2008). Unfortunately, many of the studies have not been replicated. The Bernardinelli. L et al. study showed an association between the rs28936 variant in the 3' UTR of the gene ACCN1 and MS in the isolated population of the Nuoro province. This finding was supported by the International Multiple Sclerosis Genetics Consortium who found an association close to ACCN1 3'UTR (Hafler. D et al. 2007). The rs28936 variant has now been genotyped in Britain and Finland, and the association which was observed by the Bernardinelli. L et al. study has been replicated (unpublished results). As ASICs are expressed in many of the cells which are affected during MS pathogenesis, it is logical to assume that a disruption in the expression of ACCN1 may have a contribution to the development of MS.

The experimental design used in this study has both advantages and disadvantages. The main advantage is that it clearly shows that the 3'UTR is affected by a miRNA. This experimental design clearly shows that a miRNA is binding to the 3'UTR due to the low expression of Luciferase of the control and the high expression seen when Dicer is knocked down. There is without a doubt something effecting the expression of the Luciferase, and as the only variable changed was the Dicer, it is logical to conclude that a miRNA is binding to the 3'UTR.

There are three disadvantages with this experiment. Firstly, the cell line used was MCF7. This cell line is a breast cancer cell line. This cell line was used mainly for practical reasons, for example MCF7 cells are easy to grow and they are transfected easily. They were well suited to be used in this experiment. However, as the expression of the ACCN1 gene is localised to the CNS and PNS, a neural cell line may have been more suited to the experiment. For example, miRNAs are expressed differently in different cell types, just as genes are, and as such a miRNA targeting ACCN1 may not be expressed in MCF7, as MCF7 doesn't express the ACCN1 gene. However, as MCF7 is easily transfected, the experiment was tried with it first, and the results indicate that the miRNA which targets the 3'UTR is expressed in the MCF7 cell, as we see a repression of expression.

Secondly, the negative control which was used showed a high expression of Luciferase (though not as high as the Dicer knock down) when compared with the positive control in the initial experiment (Figure 5). Logically, the negative control should show a very similar expression pattern to the positive control as the scrambled siRNA should have no effect on the miRNA

binding. This is seen frequently among transfection experiments like the one performed here. However the phenomenon is not always seen in every experiment of this kind. It has been speculated that the scrambled siRNA becomes incorporated into the RISC and out competes the miRNAs naturally present in the cell. It is also thought that the siRNAs in fact do target a gene which is involved with Luciferase activity which we don't know of, for example if an ATP related gene was targeted by the scrambled siRNA this would affect Luciferase activity as ATP is needed. Unfortunately, for both of these situations to be true, this phenomenon would be seen every time this experiment is done, but this isn't the case. As it stands this is one major drawback of using this type of experiment and until the reason for this phenomenon is found, it will continue to be a major concern for any results found using this design.

And lastly, the experiment itself found that there were three more SNPs in the 3'UTR of ACCN1 in the samples which were used. The samples which were obtained from the Bernardinelli group were from individual people. The samples were picked out of a pool of samples gathered from individuals who all showed the different SNP at the rs28936 position. Therefore it is possible that the extra three SNPs that were found during the routine sequencing after the cloning were in fact unique to the individual with whom the sample was taken. This is a possibility, and until more samples are obtained with the original SNPs, and are sequenced to discover whether the extra three SNPs found are common in patients with MS, the results from this experiment cannot be extrapolated to include all MS sufferers.

The results obtained from this study indicate a strong possibility of miRNA control in the expression of the ACCN1 gene. The first experiment showed that of the two alleles, the allele rs28936A held stronger control over the expression of Luciferase than the allele rs28936G. Due to the nature of miRNA binding, mutations in sections of the miRNA binding site such as the seed sequence or the positions 13-16 (see results section for more information on miRNA binding) can lead to a reduction in the strength of miRNA binding. This can lead to a higher level of translation and so an over expression of the protein, in this case Acid Sensing Ion channel 2 (Zhang. X and Zeng. Y 2010). As the expression difference between Dicer knock down and control was lower for rs28936G, this indicates that miRNA binding is stronger for the rs28936A allele than the rs28936G allele, and that rs28936G is therefore under weaker miRNA control and may be responsible for mis-expression of ACCN1.

Identification of three more SNPs via sequencing suggested further alteration of miRNA binding other than just the original SNP found. The results indicate that the presence of all four SNPs have equal effect on the miRNA binding. As was discussed previously, the positioning of the SNPs is very important as to whether they will affect the binding of the miRNA to its target site. The positions of all of the SNPs found in the 3' UTR of ACCN1 could therefore equally contribute to a

reduction of the miRNA binding by making the binding site less accessible to the unknown miRNA. The target site in the mRNA transcript forms a secondary stem loop and it is while in this structure the miRNA is able to bind to the transcript and suppress translation. It is possible that the SNPs cause a disruption in the formation of the stem loop structure so that the stem loop doesn't form or changes the stem loop structure slightly so that the miRNA can still bind but not as strongly. Both these situations could lead to impaired binding of the miRNA. This leads to over expression of ASIC2, which could lead to a variety of different problems with accurate cell function.

Pervious experiments which have looked at the expression of ASIC2 in different contents within the CNS give clues to potential areas in which ASIC2 over expression may lead to symptoms commonly seen in Multiple Sclerosis pathology. These studies will be discussed as follows and will be related back to ASIC2 over expression.

Studies have shown that ASIC2a is found to commonly associate with ASIC1a in the brain, including at the synapse at a 2(ASIC1a):1(ASIC2a) ratio (Zha. X et al. 2009). During this association it is currently thought that ASIC1a acts as the channel to allow the positive current (H+) to flow into the cell (in response to an increase in the extracellular pH) and that ASIC2a acts as a detector of the external pH level and activates ASIC1a (Askwith. C et al. 2004). As these two units interact so closely, it is logical to suspect that a change in the expression level of one will directly affect the delicate balance of these proteins at the synapse. An experiment performed in 2007 (Friese. M et al. 2007) which has been outlined briefly in the introduction, showed that a reduction in the expression of the ASIC1a unit (facilitated by Amiloride) lead to a decrease in axonal degradation and inflammation. As ASIC2a associates so closely to ASIC1a, it is possible that a sudden increase in ASIC2a units will facilitate an increase in ASIC1a/2a associations. As has been shown, reducing ASIC1a relieves degradation and inflammation, therefore a sudden increase in working ASIC1a/2a heteromultimers may increase degradation and inflammation, which are both a major component of the symptoms of MS. Another study which supports this theory (Chu. X et al. 2006) shows that ASIC2a helps to 'modulate' ASIC1a by activating the opening of the channel as ASIC2a is sensitive to the pH of the extracellular space surrounding the cells. ASIC2a is therefore responsible for how much of the positive current goes into the cell as it determines whether ASIC1a opens and for how long it will be open for. This ties into the theory as, if there is more ASIC2a available in the cell, then more associations can happen with ASIC1a, meaning more channels can be opened in response to extracellular pH conditions. This leads to an increase in positive current into the cell and an acidosis effect inside the cell, leading to changes in cell function like those seen in patients with MS.

Another study found that the heterodimer channel which consists of ASIC1a/2b showed novel channel properties and also contributed to acidosis induced death in neurons (Sherwood. T et al.

2011). These conclusions were drawn from an experiment in which Barium was used as an ASIC inhibitor. It is known that the presence of ASIC1a homodimer channels increases acidosis induced neural cell death; however it was found that when the ASIC1a/2b channel was inhibited by Barium, there was a lower degree of neural cell death suggesting that the heterodimer channel also has this property. Presence of an active form of the ASIC1a/2b channel causes an influx of positive current into the cell, including calcium (like the ASIC1a homodimer channels) which is known to trigger apoptosis inside the cell. Further tests with Barium showed that it directly targeted ASIC2b but not ASIC1b, which suggests that not only could Barium be used as a therapeutic drug to prevent acidosis induced neural cell death, but also that the ASIC2b subunit was a necessary component of the channel to induce cell death. These findings strongly support this theory, as acidosis and neural death are both major components of MS. It is possible (using the evidence from this study) that the SNPs in the ACCN1 gene cause an increase in the production of ASIC2b subunits within neural cells throughout the CNS and PNS. This increase leads to a higher level of ASIC1a/2b channels forming in neural cells where there is under normal circumstances none or little of these channels present. This increase could therefore lead to an increase in acidosis induced cell death (like that seen in MS patients) in neurons as there are more active channels present within the cell.

Further evidence to support this theory comes from a study which found that ASICs contribute to the activation of dendritic cells via acidosis (Tong. J et al. 2011). Dendritic cells are responsible for primary immune response in the CNS and PNS. They exist in their immature state and are still able to perform phagocytosis in the immature state which they lose once matured. Maturation happens via acidosis of the cell, which activates antigen presentation and also increases the expression of other immune genes including the MHCII region genes. In this experiment it was shown that ASIC2 (both a and b) were the only ASIC channel type to be present at the cell membrane and so it is possible that ASIC2 is a major component of the maturation of dendritic cells. The acidic extracellular environment (caused by inflammation or infection) induces the opening of the ASIC2 to activate the maturation of the cells into antigen presenting cells. It is therefore possible that an up-regulation of ASIC2 (caused by the SNPs in the miRNA binding site of ACCN1) leads to an overly high number of dendritic cells becoming matured. This could lead to high levels of cell death as the matured dendritic cells target other neural cells where they would not be targeted under regular circumstances.

Future research into this area has been suggested in order to identify the miRNA which is targeting ACCN1. It has been proposed that the λ Bacteriophage BoxB sequence can be attached to the 3’ UTR sequence and a fusion protein can be created with GFP and the λ N peptide which binds very strongly to the BoxB sequence (Pillai. R et al. 2004, Pais. H et al. 2010, Legault. P et al.

1998, Cilley. C and Williamson. J 1997). Then allowing the RISC with the miRNA in question to bind to the target site on the 3' UTR in the presence of the fusion protein will allow a complex to form in which the BoxB sequence and the N peptide will bind together, while the mRNA 3' UTR is bound to the RISC. The complex can then be purified via affinity chromatography to the GFP protein (as this is a common protein which already has strong antibodies for use in chromatography). Once the complex has been purified, the mRNA can be digested, removing the mRNA and its bound N peptide/GFP and the miRNA can be extracted from within the RISC. The miRNA can then be sequenced and identified. If this technique accurately identifies the miRNA which targets ACCN1, it will not only increase our knowledge of how the interaction is involved in the development of MS, but it will also give us a novel experiment with which to accurately identify miRNAs.

In conclusion, I have found that the ACCN1 gene is being controlled by an unknown miRNA. I also found that of the two variants discovered in the initial Bernardinelli. L et al study, rs28936A is more strongly controlled by the unknown miRNA. Lastly, I found that of all the SNPs discovered (the two originals and three 'new' SNPs) none have a significantly stronger miRNA control than the other. Further work will need to be done to verify that the three new SNPs found are not unique to the individual sample which was used in this study. Further work will also need to be done to identify the miRNA which is targeting ACCN1.

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Chapter 6: Acknowledgments

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