Gene Expression Profiling in an Alzheimer's Disease Mouse Model

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Abstract

Explaining precisely how Alzheimer's disease (AD)—the world's most common form of dementia—materializes in the human brain has proven to be one of the most elusive ends in modern medicine. Progressive memory loss, neurodegeneration, and the presence of abnormal protein aggregates of amyloid-beta (Aβ) and neurofibrillary tangles (NFT) characterize this disease. Genome sequencing provides researchers with the ability to better identify disease-related changes in gene expression, some of which may play a role in the initiation and progression toward the AD-like state. Intimate interactions between tissues have been observed in many diseases, particularly between the brain and blood. This analysis seeks to employ RNA sequencing techniques in the brain in order to identify potential drivers, molecular passengers, and significant contributors to AD, while overlaying this data with that of the blood to identify candidate genes to be used as disease biomarkers.

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Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia in the world and is characterized by progressive memory loss, neurodegeneration, and the presence of protein aggregates such as amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFT) (*1, 2*). The two major risk factors for AD are age and sex (*3, 4*). In fact, females are nearly twice as likely to develop AD than males (*5*). Diagnosing the disease has proven to be difficult because tests are primarily based on cognitive performance, not the detection of molecular indicators. The expression profile of an AD brain is substantially different from that of a normal brain, which suggests that the disease takes root from an abnormal regulation of genes (*6, 7*). Investigation of these changes in expression is a necessary next step in understanding the pathophysiology of AD.

Gene Expression Profiling

Gene expression profiling (GEP) allows for the efficient analysis of dysregulated genes in a given tissue. RNA-sequencing (RNA-Seq) is one of the most popular contemporary techniques used in GEP studies (*8*). When applying this technique to human diseases, researchers can isolate key genes that may influence the progression toward a disease-like state. This is because the AD brain exhibits significantly altered gene expression patterns when compared to a normal aged-matched brain (*6, 7*). Similarly, other tissues, including blood, also exhibit these changes in expression (*9-12*). For example, one GEP study identified 133 genes with irregular expression levels in AD blood compared to controls (*9*).

Research suggests that gene expression in one tissue can directly affect the condition of neighboring tissues (*13, 14*). In fact, this principle serves as the basis for the neurovascular hypothesis of AD, which proposes that AD pathogenesis begins with the breakdown of the blood-brain barrier. According to this hypothesis, dysregulation of various Aβ transport genes contribute to insufficient clearance of Aβ in the brain, resulting in elevated levels and subsequent formation of plaques (*13*). Research indicates that the blood-brain barrier plays a complex role in AD, likely participating in both upstream causes and downstream consequences of Aβ accumulation in the brain (*14*). In other words, the loss of function associated with a weakened blood-brain barrier results in the loss of cellular homeostasis. Despite this, individual cells within a tissue will strive to reestablish proper homeodynamic range, likely increasing or decreasing specific neuronal RNA transcripts. A host of genes, including those that code for micro RNA (miRNA), have been identified as dysregulated in either the AD brain or blood, some of which are significant in both, like that of miR-29b (*15-17*). That said, these changes in the blood might reflect changes in the AD-brain, opening the door for a potential diagnostic tool for AD from simple, non-invasive blood tests.

Currently, there is no blood-based diagnostic tool for AD. However, the association between the breakdown of the blood-brain barrier and AD grants the possibility of developing such a tool. To this end, the analysis presented here attempts to identify AD-specific changes in the brain and blood to identify candidate genes for use in a blood-based AD diagnostic tool. The development of such a tool will enhance the diagnosis of AD in early to late stages and holds the potential to identify those who have an increased genetic risk for developing AD but have yet to show symptoms.

Brief Overview of Micro RNA

Micro RNA (miRNA) are small non-coding RNA that are approximately 22 nucleotides long. Although small, miRNA play crucial roles in post-transcriptional regulation of various protein-coding genes. In fact, miRNA are estimated to be involved in the regulation of at least one-third of the mammalian genome (*18*). Since their discovery in 1993, miRNA have been shown to be key regulatory elements in various fundamental biological processes, including embryogenesis, cell differentiation, and apoptosis. Given their vital role in biological systems, it is no surprise that the dysregulation of miRNA have also been tied to various human pathologies, particularly cancers.

The mechanism by which miRNA operates requires the concerted efforts of several proteins (Appendix A). The gene encoding a specific miRNA is expressed to produce a pri-miRNA molecule in the cell's nucleus (*19*). In the nucleus, pri-miRNA is cleaved via the Drosha-DGCR8 complex to produce pre-miRNA (*20*). Exportin 5 permits the selective transport of pre-miRNA out of the nucleus and into the cytoplasm, where further modification takes place (*21*). In the cytosol, Dicer protein cleaves the pre-miRNA to create a double-stranded miRNA that ultimately unwinds to produce mature miRNA (*22*). Once unwound, the miRNA associates asymmetrically with the RNA-induced silencing complex (RISC) and directs RISC to complementary messenger RNA (mRNA) (*23*). Most miRNA form mismatched duplexes with their targets, primarily binding to the 3' untranslated region (UTR) of the target mRNA (*23*). Consequently, mismatched base pairing allows a single miRNA to have multiple targets. Once RISC has been associated with the appropriate mRNA target, post-translational regulation occurs

either by repression of translation or by direct mRNA cleavage, preventing the production of the specific protein the mRNA encoded (*24*).

MiRNA pose an interesting problem to GEP studies. Due to their small size and the absence of a poly(A)-tail, miRNA are difficult to detect in RNA-Seq analyses unless specifically targeted. Therefore, most GEP studies fail to account for miRNA in their analysis.

Challenges to Alzheimer's Disease Research

One of the primary challenges of AD research is distinguishing which factors of AD are driving the disease and which are merely passengers in AD pathology. Analysis of specific genes changing expression in AD will yield a superior understanding of the mechanisms involved in its development and may help distinguish which characteristics of the disease are molecular drivers, important contributors, or passengers in AD pathology. This investigation applies RNA-Seq techniques to help identify these important players in AD pathology by determining the expression "profile" of AD in the blood and brain. Furthermore, this project overlays these profiles with one another to determine candidate genes to be included in a diagnostic tool for AD.

Another challenge to AD-directed research is the presence of inconsistent results between labs. This issue arises primarily from three experimental caveats: (i) the type of experimental model used in investigation, (ii) tissue samples, and (iii) sex of sample population. Although animal models do provide experimental flexibility, human cohort studies are considered the "gold standard" in AD research. However, for the purpose of GEP studies, postmortem tissues would provide additional experimental freedom and practicality. In light of this, the investigation presented here employs an AD transgenic

mouse model with two human genes (APP and PSEN1) in the attempt to obtain a robust experimental model in the absence of human tissues.

Variability between labs as a result of tissue selection is common due to the variety of samples investigators may use, each having its own tissue-specific expression profile. Hippocampus, cortex, blood, and skin are commonly used, with skin in the minority (*7, 9, 25*). For the purposes of this analysis, differential expression in the hippocampus and blood were analyzed.

Inconsistencies in experimental results may arise from sex-specific changes in AD due to the involvement of sex hormones like testosterone and estrogen. In fact, estrogen is suspected to provide neuroprotective effects against the development and severity of AD (*26, 27*). This analysis utilized male AD mice and compared their expression profiles to that of control males and females. Male controls were used to minimize estrogen-dependent changes in gene expression while females were used to help identify dysregulated transcripts that are sex-independent via cross analysis. Despite this, sexual dimorphism is likely to still play a role in this investigation.

Characteristics of Alzheimer's Disease

Hallmarks of Alzheimer's Disease

The classic hallmarks of AD include progressive memory loss, neurodegeneration, and the presence of abnormal protein aggregates including Aβ plaques and NFT (*1, 2*). Sleep disturbances have also been reported in approximately 45% of cases, generally presenting as an irregularity in circadian rhythm such as excessive daytime sleepiness and insomnia at night (*28, 29*). Phenotypically, the size of an AD brain is significantly smaller than that of a healthy aged-matched brain,

predominantly attributed to atrophy of affected regions (*30*). On a cellular level, Aβ and NFT are suspected to be responsible for inducing neuronal apoptosis (programmed cell death) (*31*). Furthermore, the histological presentation of Aβ plaques and NFT are positively correlated with severity of the disease (*31-33*).

The cellular basis of AD is suspected to occur many years before a patient ever experiences symptoms. As a result, most patients are diagnosed within the later stages of the disease, making management difficult. Symptoms in the earliest stages of the disease include short-term memory loss and difficulties in concentration (*34*). Moderate stages are characterized by mood disorders, general confusion, long-term memory loss, and changes in personality (*34*). As the disease progresses into its later stages, patients begin to experience hallucinations, difficulties in motor function, and eventual loss of the brain's ability to control vital regulatory processes such as heart rate and breathing, resulting in death (*34, 35*).

Amyloid-Beta and Neurofibrillary Tangles

Aβ has been reported to play many biological roles in the normal brain, including protection against oxidative stress, enzyme phosphorylation, inflammation, neuronal differentiation, and regulation of cholesterol transport (*36-39*). In a diseased brain, however, hydrophobic fragments of Aβ are deposited in the extracellular matrix of neurons (*40, 41*). These fragments spontaneously associate with one another to form insoluble protein aggregates that ultimately result in neuronal death and subsequent neurodegeneration (*42*). Most researchers believe that the deposition of Aβ in the brain initiates the pathophysiological cascade of AD (*33*). However, other hypotheses have

been proposed overtime, with a considerable body of evidence, warranting further investigation.

One such hypothesis holds to the proposition that neurofibrillary tangles (NFTs), not Aβ, initiate the AD cascade (*43*). NFTs are primarily composed of tau protein polymers, which are responsible for the stabilization of microtubules in neurons. Microtubules are critical building blocks of the cytoskeleton. Aberrant phosphorylation of tau, such as that observed in AD, leads to the breakdown of the cytoskeleton. With respect to AD, breaking down a neuron's cytoskeleton subsequently results in the collapse of the intracellular transport system (*44*). As a result, these neurons ultimately die due to an inability to acquire and distribute appropriate macromolecules necessary for cellular functions.

Gene Expression in Alzheimer's Disease

Transcription and Gene Expression

The genes of a cell contain all the necessary information for life in the form of DNA. Through a process known as transcription, DNA is converted to messenger RNA (mRNA), which is eventually modified and transformed until being used to produce a functional protein by an additional process called translation (Appendix B). These mRNA molecules are known as "coding" RNA because they effectively code for proteins to be used by the cell. Gene expression refers to the relative activity of a particular gene, which is generally measured by the presence of the gene's mRNA product. This allows for an efficient and effective means to measure the expression level of a particular gene, including other types of RNA that do not code for proteins but may play a key role in the development of the disease.

Gene expression profiling (GEP) requires examination of the entirety of expressed genes in a given set of tissues to develop a "profile" of the cellular activity in those cells (*45*). This includes a comparison to other tissue profiles to develop a coherent understanding of cellular functions and the role of contextual cellular environments influencing the activity of the tissue(s) under investigation. To accomplish this, investigators commonly use either microarrays or next generation sequencing (NGS) techniques to get a glimpse of the totality of cellular activities in a cell from the perspective of the transcriptome (*45, 46*).

Traditionally, the cellular transcriptome is defined as the sum of all active mRNA molecules within a cell or group of cells. However, this term is commonly used in a broader sense to include all types of RNA, including non-coding micro RNA (miRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA) among others. Thus, disease-focused studies that employ these techniques are intended to assist investigators in identifying areas of the transcriptome that display abnormal patterns and examine certain genes either responsible or associated with the disease.

Next Generation Sequencing (NGS)

NGS allows for the rapid sequencing of the transcriptome to determine differential expression profiles in a fast and efficient manner at a relatively low cost (*47*). Furthermore, NGS permits the identification of novel RNA isoforms and splicing sites largely because the technique does not rely heavily on isoform-specific probes like traditional methods (*48*). NGS is synonymous with RNA-Seq because this technique directly sequences the RNA in a given group of cells, providing researchers with the ability to determine which genes are active versus inactive and to what degree.

Regulation in Alzheimer's Disease

Gene activity and subsequent expression can be modulated by several factors. In particular, epigenetic factors affect the expression profile of a given tissue by modifying nuclear DNA in such a way that gene expression is affected without changing the genetic code. A considerable amount of research has identified many genes in AD to be epigenetically modified by chemical markers such as cytosine methylation. Whether or not a particular gene is expressed within a cell or group of cells is determined by these chemical markers. This phenomenon is heritable and has been shown to be influenced by environmental stressors (*49*). That said, epigenetic changes may be the driving force behind many of the observed AD-related changes in gene expression. Although establishing the epigenetic basis of AD is not within the scope of this analysis, many of the observed changes in gene expression are likely to have some basis in the modification of epigenetic markers.

AD does not follow a traditional pattern of inheritance. Several studies have identified epigenetic changes in the DNA of AD patients (*50-53*). Additionally, multiple studies have identified epigenetic changes in genes that code for miRNA responsible for critical regulatory processes in the cell (*54-56*). Furthermore, several miRNA have been previously identified as playing a key role in the progression of AD (*57*). This connection highlights the importance of epigenetic changes in the development of AD and warrants further investigation into the potential causative role of epigenetically modified miRNA in AD pathophysiology.

Experimental Design and Rationale

5XFAD Mouse Model

Mice are a common scientific model for understanding the complexities of human pathogenesis. Because they share similarities in much of their genome, mice exhibit many of the same diseases as humans (*58*). Using a mouse model allows for greater flexibility of study and thus allows investigators to obtain a greater analysis of a particular disease that otherwise would have been unethical in humans. Transgenic mouse models are frequently used because they provide researchers with the ability to selectively modify the organism's DNA to produce a particular disease or disease-like state, often by inserting human DNA in the mouse genome.

This investigation utilized the 5XFAD transgenic mouse strain. This strain contains three human mutations in the amyloid-beta precursor protein (APP), including the Swedish mutation (K670N/M671L), Florida mutation (I716V), and the London mutation (V717I). Furthermore, the 5XFAD mouse model contains two human mutations in the gene encoding presenillin 1 (PSEN1), including M146L and L286V designed to overexpress mutant PSEN1 in neuronal tissue. Collectively, these five mutations are associated with familial Alzheimer's disease (FAD) in humans and work synergistically in the accumulation of Aβ plaques. The APP and PSEN1 genes are both regulated via the mouse Thy1 promoter, designed to aggressively overexpress these genes in the brain. As a result, the 5XFAD strain elicits the characteristic features of human AD pathology in mouse nervous tissue, including neurodegeneration, Aβ plaques, and NFT. The 5XFAD mouse strain provides a robust AD transgenic mouse model that results in the

development of Aβ plaques at 2 months, cognitive impairment at 4 months, and neuronal apoptosis at 9 months of age (*59*).

RNA-Sequencing

This analysis utilized NGS to obtain a comprehensive, genome-wide analysis of gene expression patterns in the 5XFAD mouse strain using two different tissues: hippocampus and blood. Progression of AD is well documented in the hippocampus and its effects on the tissue are well-known. Furthermore, there is a strong correlation between severity of disease and presence of characteristic AD features in this region of the brain, specifically Aβ plaques, NFT, and cognitive decline (*60, 61*). Blood tissue was also collected to investigate the role of the blood-brain barrier in AD pathology and to use this data to determine candidate genes for an AD diagnostic tool.

Several studies highlight a parallel correlation between gene expression changes in the brain and blood (*11, 17, 62, 63*). Furthermore, a considerable amount of evidence suggests an intimate interaction between the AD brain and peripheral leukocytes (*9, 10, 64-66*). Transcription factors that are involved in cardiovascular disease have also been identified as being dysregulated in the AD brain (*67, 68*). These studies suggest a possible involvement of tissue-specific crosstalk between the blood and brain that may be utilized to identify genetic risk factors or disease-contributing genes in the brain from simple, non-invasive blood tests.

The following was done in collaboration with Amanda Házy: RNA was isolated and purified from the brain and blood of nine mice (3 5XFAD AD males, 3 normal males, and 3 normal females) for comparison. Purified RNA was then reverse transcribed into cDNA and sequenced using an Illumina MiSeq gene sequencer. Expression data

were then normalized to control mice to determine significantly dysregulated genes in both tissues. Ten significant genes were selected to be analyzed in the brain based on p-value (≤ 0.05) and tested using RT-qPCR. Gene-by-gene confirmations were normalized using endogenous controls (Tubb3 and Gapdh).

Selection of Endogenous Control Genes

Suitable endogenous control genes were selected based upon the following criteria: (i) high expression in control and AD samples to ensure detection, (ii) minimal variation between those samples to increase experimental consistency as (relatively) constitutively expressed genes, (iii) gene primers exhibited melt curves with single peaks to minimize qPCR side reactions, and (iv) genes displayed primer efficiencies consistently near 1 to maximize uniform amplification cycles. Tubb3 (beta-tubulin 3) was shown to be a sufficient control for the purposes of this analysis, although the relative abundance in the blood of normal males was not ideal. Gapdh (glyceraldehyde-3-phosphate dehydrogenase) was also shown to be a suitable control gene for AD male v. normal female (brain and blood), however its adventitious expression in the brain of normal males made it insufficient as an endogenous control gene in male hippocampal tissue.

Micro RNA Analysis

Several studies have identified a correlation between dysregulated miRNA and the AD-like state, many of which display epigenetic modifications (*56, 69, 70*). For the purposes of this study, miRNA with demonstrated epigenetic modifications were analyzed using a miScript PCR primer assay (Qiagen) to determine relative expression patterns of miRNA compared to that of endogenous controls, Tubb3 and Gapdh. MiR-17,

26b, 92, and 702 have been previously identified by our group as being epigenetically modified in terms of methylation status (data not shown).

Experimental Methods

Mouse Euthanasia

Three male 5XFAD mice were used for this analysis and data from these mice were normalized using three normal males and three normal females. Mice were euthanized with carbon dioxide and sacrificed by decapitation in accordance to the Institutional Animal Care and Use Committee (IACUC) accepted protocol. Once euthanized, mouse tissues were obtained for RNA isolation.

RNA Isolation

RNA was isolated from the hippocampus and blood of each mouse. To accomplish this, the hippocampus was dissected and homogenized in 1 mL Trizol per 50-100 mg tissue (Invitrogen). Blood was obtained via cardiac puncture and placed in EDTA tubes for subsequent centrifugation (10 minutes) to isolate RNA-rich leukocytes. Subsequently, 100 μL of cells per sample were then homogenized in 1 mL Trizol. After homogenization in Trizol, tissues were incubated at room temperature for 5 minutes followed by the addition of 0.2 mL chloroform per 1 mL Trizol. Samples were then subjected to centrifugation (12,000 x g for 15 minutes) before removal of the RNA-rich aqueous phase. RNA precipitation was performed by adding 0.5 mL of isopropanol per 1 mL of Trizol for 10 minutes at 4 ˚C. RNA was pelleted by centrifugation at 12,000 x g for 10 minutes. The precipitate was washed with 75% ethanol and centrifuged at 12,000 x g for 5 minutes. Samples were then allowed to dry and resuspended in 50 μL DEPC-treated water.

cDNA Preparation

A MiScript II RT kit (Qiagen) was used for cDNA preparation. Conversion to cDNA and subsequent purification was accomplished by following manufacturer's Hiflex protocol to obtain a final cDNA concentration of 2.5 ng/μL.

For comparison to the MiScript protocol, an iScript cDNA Synthesis kit (BioRad) was also used to test the reliability and sensitivity of each kit. A final cDNA concentration of 2.5 ng/μL was synthesized from RNA according to the manufacturer's protocol.

RNA Sequencing and Analysis

The following RNA Sequencing methods were done in collaboration with Amanda Házy: Each cDNA sample was prepared and sequenced using an Illumina MiSeq sequencer, according to manufacturer's protocol. Sequencing quality was assessed using the Galaxy platform. FastQ files were imported into the Galaxy server for differential expression analysis and the FastQ Groomer tool was used to prepare data for downstream analysis using the Tuxedo suite. FastQ files were aligned to the mm9 reference genome using TopHat and BowTie. Two separate FastQ files resulted and were combined through the TopHat/BowTie software.

Cufflinks software was used to assemble transcripts and determine FPKM value (fragments per kilobase of transcripts per million mapped reads). Furthermore, a maximum intron length of 300,000, minimum isoform fraction of 0.1, and a pre-mRNA fraction of 0.15 was selected using the Cufflinks software. Additionally, various corrective analyses were used to improve statistical significance (quartile normalization, bias correction, multi-read correction, and effective length correction). The UCSC

RefFlat table for the mm9 mouse genome was used as a reference annotation for Cufflinks, which allowed for differential transcript expression, splicing, and promoter use. Cuffmerge was used to combine accepted hits from TopHat to align AD samples to background. Cuffdiff and Cufflink files were then converted to csv format to allow compatibility of sequencing results with Microsoft Excel. Using these files, CummeRbund software was employed to visualize data and gene ontology was assessed using GeneCodis (*71-73*).

RT-qPCR Confirmations: miRNA and mRNA

RNA isolated from the hippocampus of AD males and normal females was converted to cDNA and analyzed using a MiScript RT II kit (Qiagen), according to manufacturer's protocol for HiFlex (mRNA and miRNA) and HiSpec (miRNA only) detection of transcripts by qPCR. Data were then analyzed, normalized, and converted to $\log_2$ fold change to visualize the direction and significance of dysregulation.

Results

RNA-Sequencing Analysis

AD male versus normal female. RNA-Seq analysis identified 2250 genes to have demonstrated changes in gene expression in the hippocampus of AD males when compared to that of normal females (Figure 1). Of these genes, 230 were significantly dysregulated in the AD hippocampus (Figure 1A). Statistical significance was determined using a p-value ≤ 0.05. In the AD blood, 58 genes were identified to be dysregulated and 8 were shown to be significant in both the brain and blood of AD (Figure 1B).

AD male versus normal male. The second RNA-Seq ananlysis identified 729 genes displaying significant changes in gene expression in the male AD hippocampus

when compared to that of normal males (Figure 2). These genes were determined to be statistically significant as assessed by a p-value of ≤ 0.005. Similarly in the blood of AD, 151 genes were determined to exhibit expression changes compared to that of normal blood. Of these genes, 75 were revealed to have been statistically significant expression in both the brain and blood.

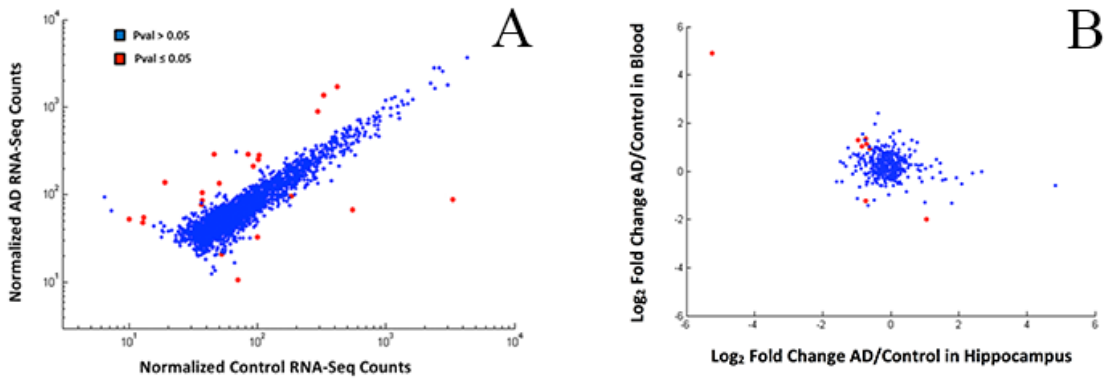

Figure 1. Differential expression in AD hippocampus and blood in AD male/female control. (A) AD male hippocampus and blood were normalized to that of a normal female revealing 2250 dysregulated genes, 230 of which were significantly dysregulated. P-value > 0.05 is illustrated in blue while those with a p-value ≤ 0.05 are depicted in red. Twenty-four genes demonstrated significant expression changes in the AD hippocampus (red) compared to that of control mice. (B) RNA-Seq data from AD male and normal female are plotted on the x and y axis for each tissue under investigation (hippocampus and blood, respectively). 8 genes were shown to be significantly dysregulated in both the brain and blood (red). Figure courtesy of Amanda Házy.

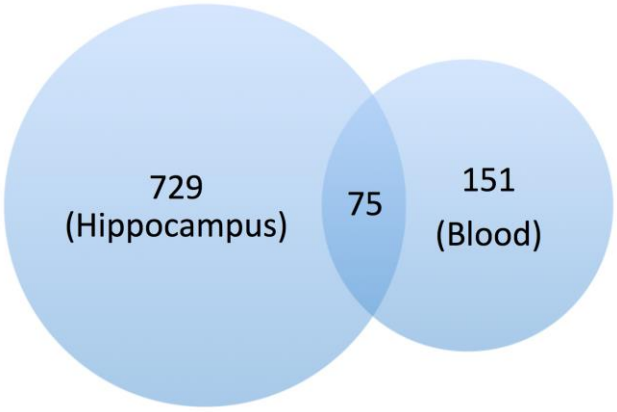

Figure 2. Differential expression in AD hippocampus and blood in AD male/male control. RNA-Seq in AD male/male control revealed 729 genes that significantly (p-value ≤ 0.005) dysregulated in the brain while only 151 were identified in the blood. The union of these genes revealed 75 that are significant in both tissues.

Comparative RNA-Sequencing analysis in brain. Comparative analysis of differential RNA-Seq data in the brain across sexes revealed 4 genes that displayed significant changes in gene expression in AD males when compared to that of both normal males and females (Figure 3). Statistical significance was assessed based on p-value ≤ 0.0005. The genes Ctsz, Hexa, Tyrobp, and Laptm5 were all observed to be up-regulated in both analyses. Gene ontology of these genes revealed immune cell regulation and differentiation, proteolysis, and inflammation as major biological processes that are affected in the AD brain (Table 1).

Comparative RNA-Sequencing analysis in brain and blood. Genes that were found to be dysregulated in the AD male/control female analysis were compared to those found in the AD male/control male analysis to determine sex-independent changes in AD blood and brain. Significance was determined using a p-value ≤ 0.05. One gene, Hmgn1 (high mobility group nucleosomal binding domain 1), was observed to be significant in all tissues, regardless of the sex used for normalization. Gene ontology of Hmgn1

revealed an involvement in gene expression and cellular differentiation by association with nucleosome binding (*74*).

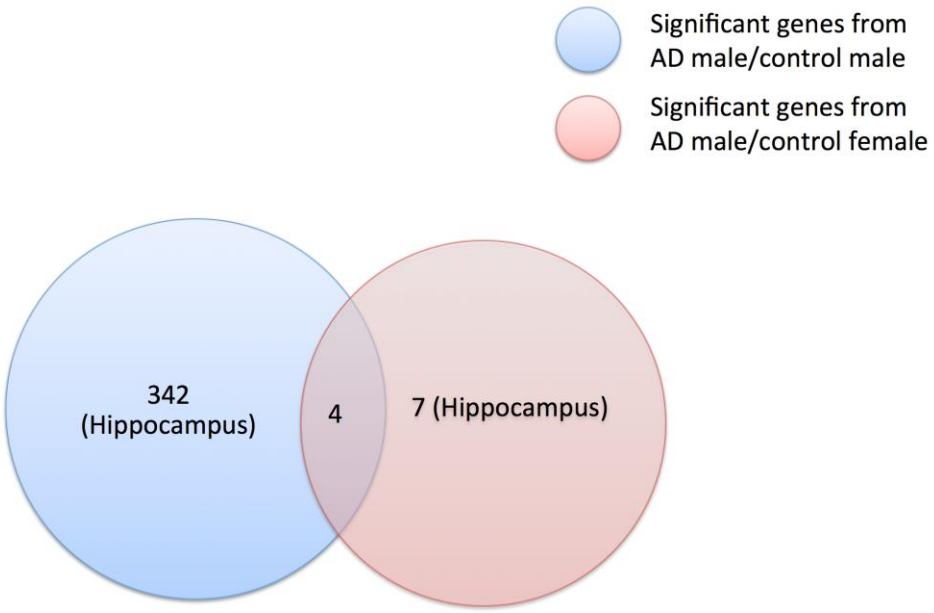

Figure 3. Comparative RNA-sequencing analysis in hippocampus. Significant (p-value ≤ 0.0005) genes in the hippocampus of the AD male/male control analysis were overlaid with those found to be significant in the AD male/female control analysis. The union of these two lists revealed 4 genes (Ctsz, Hexa, Tyrobp, and Laptm5) that were significantly dysregulated regardless of the sex by which the data were normalized.

Symbol	Name	Regulation (Up/Down)	AD Connection	GO Category
Tyrobp	Tyro protein tyrosine kinase-binding protein	Up	Yes (*75-77*)	Immune cell regulator
Hexa	Hexosaminidase subunit alpha	Up	Yes (*78-80*)	Cell differentiation, Lysosome organization, Myelination
Ctsz	Cathepsin z	Up	Yes (*81*)	Proteolysis
Laptm5	Lysosomal protein transmembrane 5	Up	Yes (*82, 83*)	Inflammation

Table 1. Gene ontology and significance in differential brain expression. Gene ontology of the 4 intersecting genes from RNA-Seq analysis in the brain was retrieved using GeneCodis (*71-73*). Ontological analysis revealed cellular regulation, differentiation, proteolysis, and inflammation as major biological processes that are affected in AD. Regulation Up/Down represents the relative abundance in transcript availability in AD compared to that of healthy controls.

Micro RNA Analysis

Of the genes previously identified by our group to be epigenetically modified, miR-17 displayed significant dysregulation in the AD hippocampus when compared to endogenous controls (Figure 4). An average $\log_2$ fold change of 2.16 (n=3) was observed in miR-17, demonstrating significant up-regulation. In contrast, miR-26b, miR-702, and miR-92 did not show significant changes with respect to gene expression despite their reported changes in methylation status.

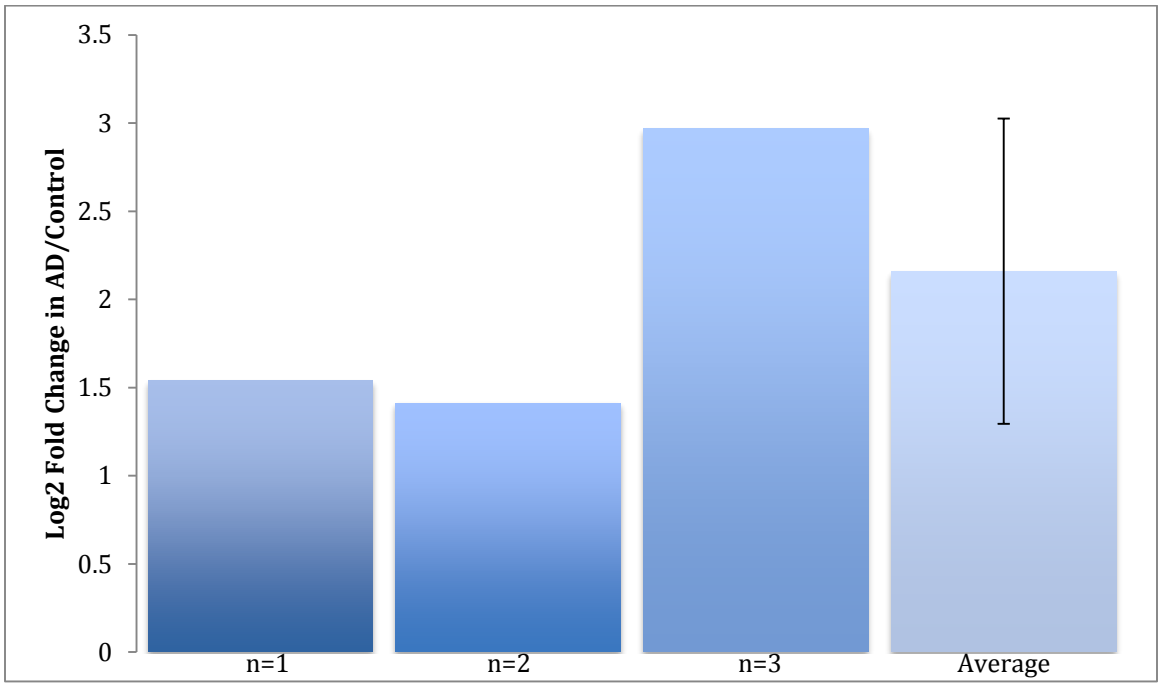

Figure 4. Significant up-regulation of miR-17. Gene expression levels of epigenetically modified miR-17 was identified using the miScript primer assay in triplicate (3 AD males vs 3 normal females) and normalized using the endogenous controls Gapdh and Tubb3. On average, miR-17 was identified to exhibit a fold change of 2.16 (1.54, n=1; 1.41, n=2; and 2.97, n=3).

Gene-by-Gene Confirmations via qPCR

The MiScript (Qiagen) and iScript (BioRad) cDNA synthesis kits were compared side-by-side to investigate reliability and sensitivity of cDNA synthesis. Relative abundance of RNA transcripts as high or low count transcripts may result from unintentional differences in cDNA amplification efficiencies associated with each kit. That said, the selected cDNA synthesis protocols were compared with negligible differences between them (data not shown), strengthening the reliability of subsequent confirmations.

Gene-by-gene confirmations were conducted via qPCR on 10 randomly selected genes that showed up as significant in the RNA-Seq AD male/normal male analysis. Of these genes, three were confirmed as being significantly changed in the AD hippocampus

when compared to that of normal male controls: Ercc2, Grn, and Ppia (Figure 5). Log_2 fold change for each was assessed to determine direction of dysregulation and was found to be -0.81, 2.4, and -1.1 for Ercc2, Grn, and Ppia respectively.

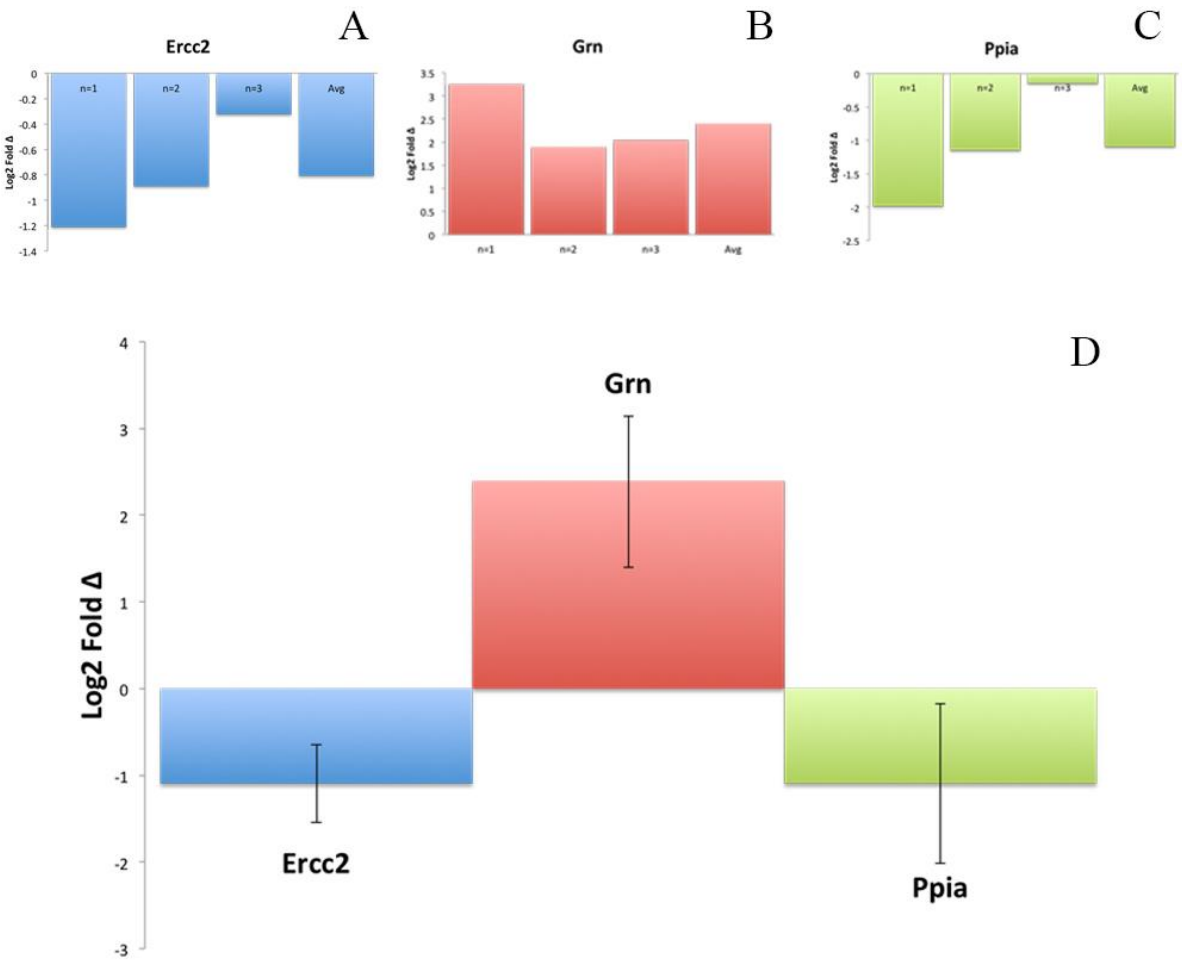

Figure 5. Gene-by-gene confirmations via qPCR in AD hippocampus. Of the 10 genes chosen for this analysis, 3 confirmed reproducibly (n=3) via qPCR (Ercc2, Grn, and Ppia). (A) Ercc2 (blue) demonstrated a consistent down-regulation with an average log2 fold change of -0.81. (B) Grn (red), on the other hand, was found to be up-regulated in AD, exhibiting an average log2 fold change of 2.4. (C) Ppia (green) also confirmed to exhibit gene expression changes and was shown to be down-regulated in AD (log2 fold change of -1.1). (D) qPCR analysis confirmed significant gene expression changes in Ercc2 (blue), Grn (red) and Ppia (green).

Discussion

Up-regulation of miR-17 and Apoptosis

This study has demonstrated miR-17 to be significantly up-regulated in the male AD hippocampus. Given that miRNA regulation occurs at the post-transcriptional level, a dysregulation of miRNA subsequently modulates the expression levels of its targets. That said, an analysis of miR-17 targets revealed genes that are responsible for foundational biological processes, including those that are involved with neuronal apoptosis and cellular proliferation. Several studies have suggested that early activation of apoptotic pathways in the brain contribute significantly to the pathophysiology of AD.

The anti-apoptotic protein Bcl-2 (B-cell lymphoma 2) is involved in regulating apoptosis and has been shown to be a target of miR-17, inducing autophagy and subsequent apoptosis (*84, 85*). RNA-Seq data also revealed Bcl-2 to be down-regulated in the AD brain when compared to controls (data not shown). Furthermore, one study found that overexpressing Bcl-2 in a transgenic AD mouse model not only increased active memory in mice, but also reduced the levels of Aβ plaques and NFT in the brain (*85*). Thus, the targeting of Bcl-2 by miR-17 may promote apoptosis in the AD brain and ultimately result in neuronal atrophy.

BMPR2 (bone morphogenic protein receptor type 2) is involved in regulating neuronal apoptosis and has been reported as a miR-17 target (*86*). Indeed, RNA-Seq analysis revealed BMPR2 to be down-regulated in the AD brain (data not shown). Furthermore, one study showed that inhibition of BMPR2 induces apoptosis in some tissues (*87*). Thus, miR-17 may play an active role in AD by mediating the inhibition of BMPR2, likely contributing to early activation of apoptotic pathways such as caspase-3.

MiR-17 has also been reported to target E2F, a family of key regulatory transcription factors (TF) that are responsible for providing a cell with the means to advance beyond the G1/S-phase checkpoint of the cell cycle (Appendix C). Several studies have also identified an E2F-dependent apoptotic pathway that recruits many of the same pro-proliferative genes activated in cell cycle progression (*88, 89*). E2F ultimately induces the transcription of pro-apoptotic genes, initiating the first step of catastrophic neuronal death in response to chemical stimuli (*88*). To complicate matters further, some studies have reported that miR-17, in fact, promotes intracellular proliferation of the E2F family, despite being one of its targets (*90*). A model proposed by Cloonan and colleagues attempts to reconcile these seemingly contradictory results by demonstrating that the miR-17 family can behave differently depending on the cellular context. In other words, the activity of the miR-17 family is driven by the relative affinity and concentrations of its targets within the cell (*91*). This is obtained by virtue of the fact that miR-17 also targets several anti-proliferative transcripts with a greater affinity than that of E2F, subsequently increasing its intracellular levels (*90*).

Furthermore, the E2F-dependent apoptotic pathway is driven by trophic factor deprivation (cellular starvation), which is known to be associated with the collapse of the cytoskeleton in response to the formation of NFT (*44, 89*). Taken together, these observations suggest miR-17 may play a role in the E2F-dependent apoptotic pathway by inhibiting anti-proliferative molecules that may interfere with responses to a pro-apoptotic stimulus (Appendix D). Although little is known about the association of the E2F-dependent pathway in AD, much less the role of miR-17 in this pathway, the data presented here highlights the potential involvement of this pathway in AD pathogenesis.

Previous work by our lab has demonstrated that the gene encoding miR-17 is hypomethylated in the AD hippocampus, affecting the relative abundance of this miRNA in the tissue. This analysis has confirmed the significant up-regulation of miR-17 via qPCR in the AD hippocampus. In light of the information mentioned above, epigenetic changes in gene expression appear to play a significant role in the pathophysiological progression of AD.

Down-regulation of Ercc2

The gene Ercc2 (XPD; excision repair cross-complementation group 2) is responsible for transcription-coupled nucleotide excision repair, which allows a cell to "proof read" and edit misplaced nucleotides in its DNA (mutations) before passing this information on to its progeny (*92*, *93*). Furthermore, failure for Ercc2 to respond to mutations in the DNA appropriately results in an increased frequency of apoptosis (*93*). The data presented here reveal that Ercc2 is slightly down-regulated in our model (log2 fold change of -0.81). Thus, it is likely that the differences in gene expression of Ercc2 may contribute to the cell's reduced ability to prevent neuronal apoptosis, further contributing to the development of AD in our model.

Up-regulation of Grn

Grn (granulin) is a growth factor that is involved in the positive regulation of neuron projections such as axons and dendrites. Current research suggests that mutations in Grn translate to an increased risk for development of various forms of dementia, including AD (*94-97*). However, Grn likely plays a role as a disease-modifying gene as opposed to a causative biochemical role in AD pathology, especially with respect to sporadic development (*98*). Furthermore, one study showed that variations in the Grn

gene are not even major contributors to late on-set AD (*99*). That said, the datum presented here is consistent with other mouse models in that it indicates that Grn is significantly up-regulated (log2 fold change of 2.4) in the AD brain (*97*). Increased Grn gene expression is suspected to result from a corrective cellular response to AD pathology, as other labs have suggested (*98*).

Down-regulation of Ppia

Ppia (CypA; peptidylprolyl isomerase A) assumes many biological roles in the brain, most significantly neuronal differentiation and regulation of apoptosis (*100, 101*). The data presented here reveal that Ppia is down-regulated in this model, suggesting a disruption of these processes in the brain. Specifically, Ppia has been identified as a necessary component of retnoic acid (RA) neuronal differentiation (*101*). Thus, these data suggest that the ability of the AD brain to differentiate has been diluted due to a decreased availability of this protein in the cytosol, leading to reduced plasticity of these cells that may contribute to AD. Additionally, Ppia has been shown to initiate the production of the antiapoptotic protein, Bcl-2 (*102*). This suggest that the AD brain's ability to resist apoptosis is further reduced because of the down-regulated nature of Ppia. The collective effects of miR-17 and Ppia on Bcl-2 demonstrate the importance of Bcl-2 protein in the pathophysiology of AD. Furthermore, the weakened ability of the AD brain to undergo differentiation results in insufficient neuronal specificity.

Differential RNA-Sequencing Analysis

Individual RNA-Seq data files (AD male vs. normal female, AD male vs. normal male) resulted in major differences by which genes were considered "significant." One explanation is that males and females are highly dimorphic with respect to the regulation

of their genomes. Specifically, differential expression of steroid hormones, particularly estrogen and testosterone, plays a significant role in gene expression and thus influence GEP studies (*103-106*). The primary goal of the differential RNA-Seq analysis was to determine candidate genes to be used in the development of a diagnostic tool for AD. Thus, any potential biological markers for AD that are greatly affected by the relative expression of estrogen and testosterone are insufficient for the purpose of a non-sex-specific diagnostic tool.

Several genes were observed to be dysregulated in the brain and blood of AD mice, indicating that a breakdown of the blood-brain barrier may preclude the development of the familial AD-like state. Hmgn1 was the only gene observed to be dysregulated in both tissues, regardless of the sex used to normalize the data. Furthermore, this analysis is the first to identify Hmgn1 as being dysregulated in the brain and blood of AD. Although the involvement of Hmgn1 in AD is poorly defined, its candidacy as a biological marker is solely contingent on the nature of its dysregulation (Figure 6). Thus, Hmgn1 reveals itself to be a suitable candidate as a predictive biomarker for AD.

The differential analysis of the AD brain revealed four significantly ($p\text{-value} \leq 0.0005$) dysregulated genes, all of which have been previously associated with AD to some extent (Table 1). These genes are involved in several processes, notably inflammation, differentiation, and immunological responses. Most importantly, these genes are not sex-specific. Thus, their dysregulation is not ultimately dependent on hormonal regulation. That said, this analysis adds to the growing body of evidence suggesting the importance of these genes in AD pathophysiology.

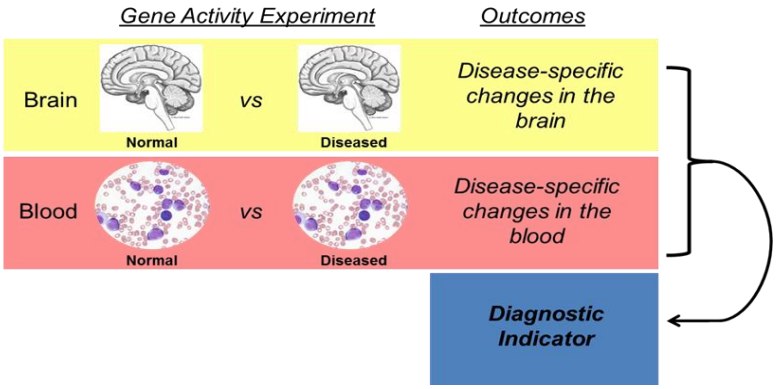

Figure 6. AD diagnostic indicator from GEP studies. Disease-specific changes in the blood and brain could be utilized in a diagnostic test for AD. Significantly dysregulated genes in each tissue can be assessed using a simple, non-invasive blood test that parallels changes in the brain leading to an effective and cost-efficient diagnostic tool.

Conclusion

AD is one of the greatest medical and social challenges of this generation. Although the disease had been characterized for over 100 years, AD-directed therapies today provide virtually no clinical improvement to those who suffer from this disease. This issue stems from the fact that very little is known about the pathophysiology of AD. A comprehensive understanding of AD necessarily entails two primary discoveries: (i) elucidation of genetic drivers, important contributors, and mere passengers of the disease, and (ii) effective, systematic diagnostic means.

This investigation adds to the growing body of evidence that suggest Tyrobp, Hexa, Ctsz, and Laptm5 are central to the progression of AD while also highlighting additional genes that seem to play vital roles in the progression toward the disease state. Mir-17 plays a critical *prima facie* role as a genetic driver of AD pathophysiology, particularly because of its responsibility in targeting regulatory genes involved in neuronal apoptosis and proliferation in addition to its demonstrated epigenetic status. Additionally, this analysis identified four probable important contributors to AD

pathology: Bcl-2, BMPR2, E2F, and Ercc2. Furthermore Grn was highlighted in this analysis, displaying significant dysregulation likely due to its role as a passenger of AD, not a contributor.

Current diagnostic means for AD are not only ineffective but are also costly and invasive. Improved understanding of the blood-brain barrier in AD yields the potential for the development of a multi-gene diagnostic "cocktail" that serves as a predictive biological marker for AD. Hmgn1 was shown in this analysis to be significantly dysregulated in both the blood and brain of AD, regardless of sex. Thus, Hmgn1 is a strong candidate gene for incorporation into a diagnostic tool of this nature.

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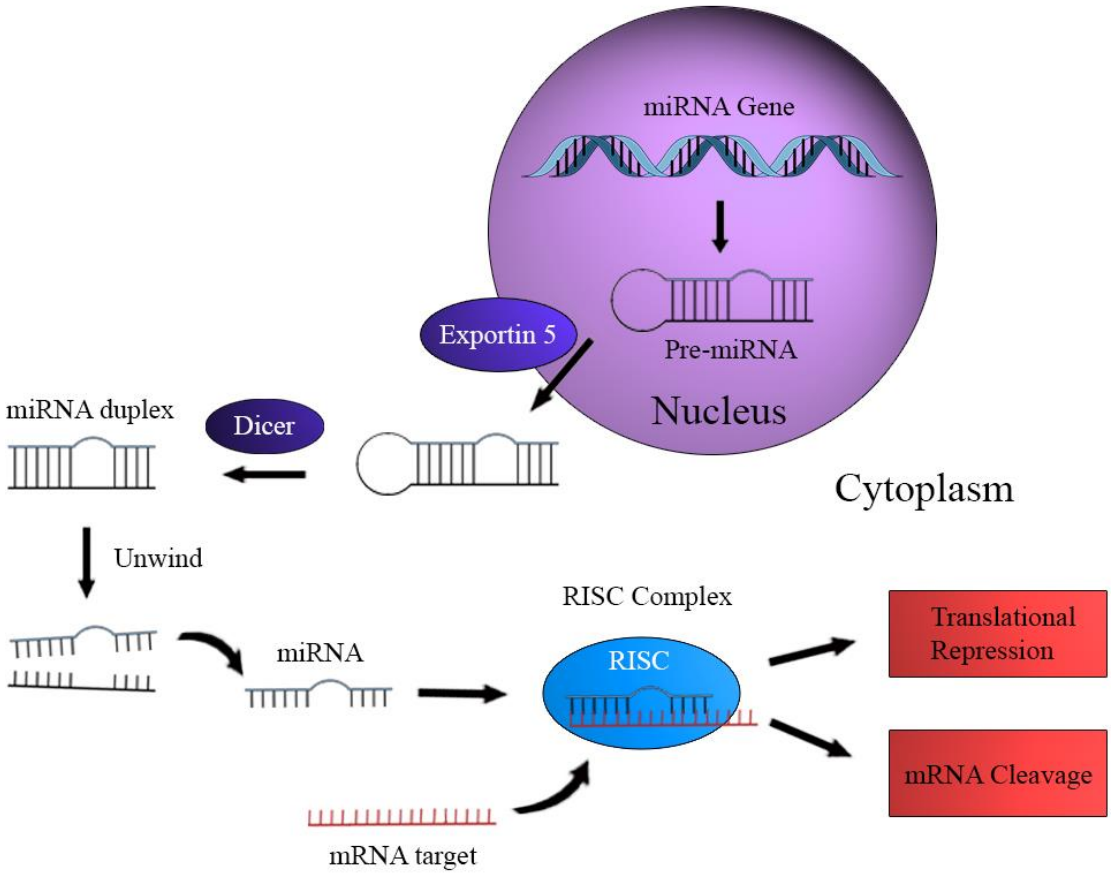
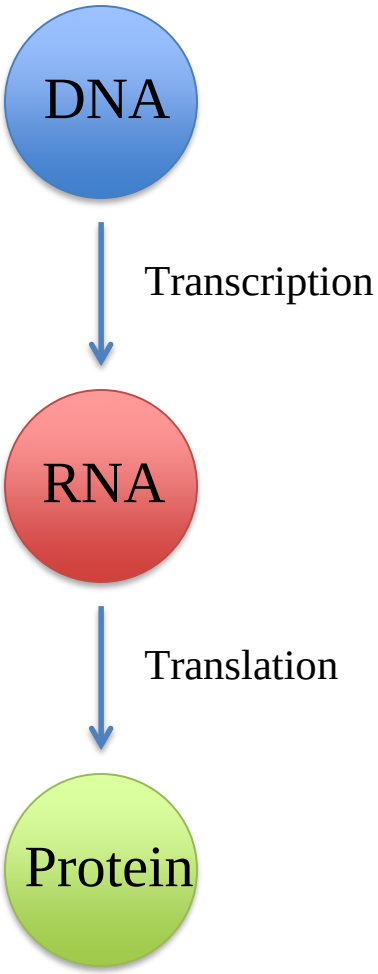
Appendix A



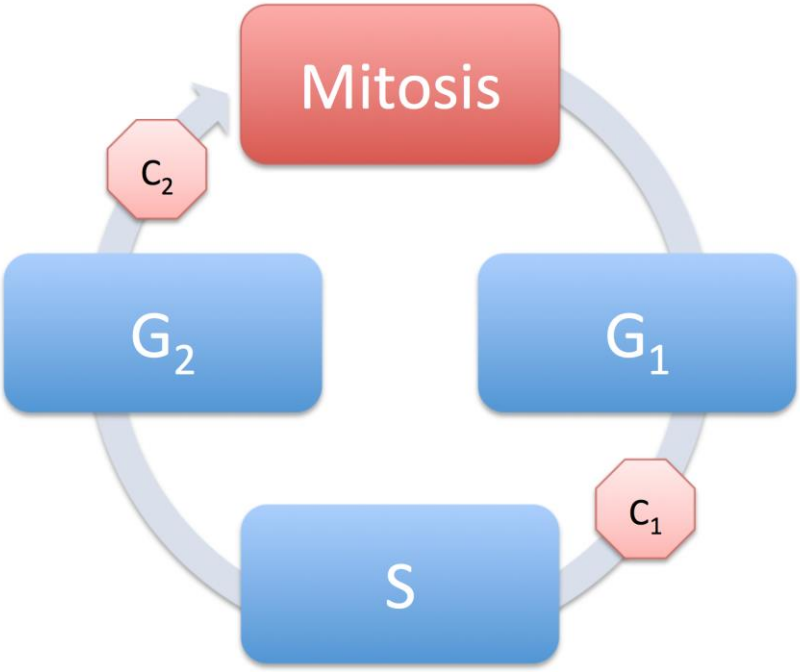
The miRNA mechanism. A gene that encodes an miRNA is transcribed to produce a pri-miRNA molecule, which is then converted into a pre-miRNA via the Drosha protein (not shown). Pre-miRNA is then exported into the cytoplasm where Dicer cleaves the pre-miRNA into a double-stranded RNA fragment (miRNA duplex). The fragment then unwindes, becoming a mature miRNA. The miRNA associates with RISC and its target mRNA to create the RISC complex, either cleaving the target mRNA directly or repressing its translation.

Appendix B



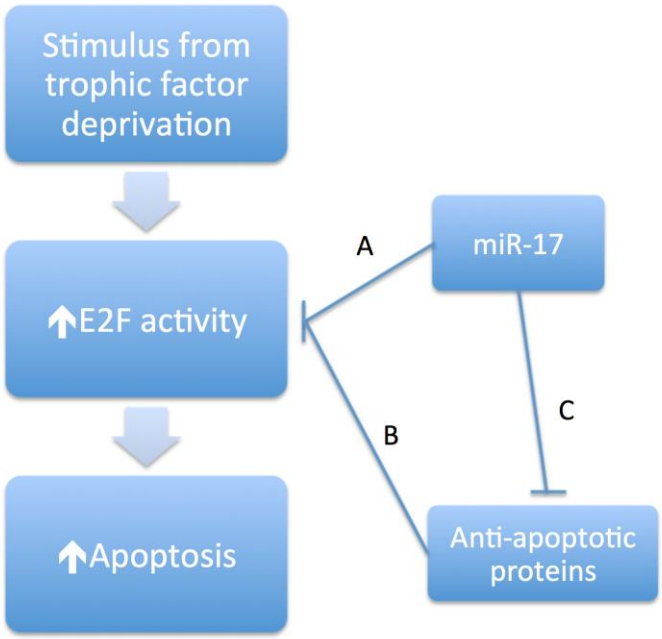
Central dogma of biology. Chemical information is stored in the form DNA (blue) in the nucleus of a cell. This information is converted into RNA (red) by a process called transcription. RNA is then modified and translated into protein (green), the chief actor of the cell. Next generation sequencing (NGS) often utilizes the accessibility of RNA, typically mRNA, to infer either the relative abundance of a protein, transcriptional activity of the gene, or both within a given cell.

Appendix C



The cell cycle. Active cells must progress through a series of definite stages before cellular proliferation (i.e. division) may take place. These stages consist of interphase (blue) and mitosis (dark red). Interphase can be further divided into three sub-phases: G_1, S, and G_2. As the cell cycles through these stages in preparation of mitosis, two cellular "checkpoints" must be successfully passed (C1 and C2, light red). In order to pass C1, free E2F must be present in order to initiate transcription in preparation for S-phase.

Appendix D



Regulation of the apoptotic E2F-dependent pathway. Stimulus via trophic factor deprivation promotes the activation of the apoptotic E2F-dependent pathway, which responds by increasing the transcription of pro-apoptotic genes. (A) Under normal conditions miR-17 targets E2F, regulating its activity by silencing the transcript to keep it within a normal homeodynamic range. (B) Anti-apoptotic proteins are also responsible for keeping E2F within homeodynamic range. However, miR-17 exhibits an increased affinity (C) for the transcripts of these anti-apoptotic proteins. Thus, under the correct intracellular conditions, like that in the activation of the apoptotic E2F pathway, miR-17 can have a proliferative effect on E2F activity by virtue of the fact that it is "distracted." This complex regulation pathway illustrates the importance of maintaining each molecular component of a cell to ensure proper cellular functionality.

Appendix E

From: Amanda Hazy <amanh15@vt.edu>
Sent: Sunday, May 8, 2016 8:15 PM
To: Dalton, Matthew R
Subject: Re: Graph permission

Matt,

You have my permission to use those graphs in your thesis. Enjoy the rest of your semester!

Amanda

On Sat, May 7, 2016 at 5:29 PM, Dalton, Matthew R <mdalton15@liberty.edu> wrote:

Hi Amanda,

I hope this message finds you well. As a requirement of the Digital Commons, I am requesting written permission from to include a couple of your graphs in my honors thesis:

Fig 1. "Differential expression in AD hippocampus and blood in AD male/female control."
Fig 6. "AD diagnostic indicator from GEP studies."

I have attached a copy of my thesis for your convenience.

All the best,

Matt Dalton