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Sporadic Alzheimer's disease fibroblasts display an oxidative stress phenotype

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Abstract

Alzheimer's disease (AD) is a major health problem in the US, affecting one in eight Americans over the age of 65. The number of elderly suffering from AD is expected to continue to increase over the next decade, as the average age of the US population increases. The risk factors for and etiology of AD are not well understood; however, recent studies suggest that exposure to oxidative stress may be a contributing factor. Here, microarray gene expression signatures were compared in AD patient-derived fibroblasts and normal fibroblasts exposed to hydrogen peroxide or menadione (to simulate conditions of oxidative stress). Using the 23K Illumina cDNA microarray to screen expression of > 14,000 human genes, we identified a total of 1017 genes that are chronically up- or down-regulated in AD fibroblasts, 215 of which were also differentially expressed in normal human fibroblasts within 12 h after exposure to hydrogen peroxide or menadione. Pathway analysis of these 215 genes and their associated pathways reveals cellular functions that may be critically dysregulated by oxidative stress and play a critical role in the etiology and/or pathology of AD. We then examined the AD fibroblasts for the presence of oxidative DNA damage and found increased accumulation of 8-oxo-guanine. These results indicate the possible role of oxidative stress in the gene expression profile seen in AD.

Graphical abstract

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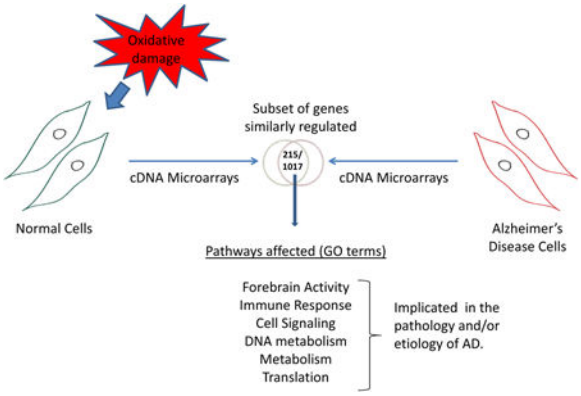

Introduction

Alzheimer's disease (AD) is a complex, heterogeneous disorder primarily affecting the elderly, characterized by progressive loss of neuronal cells and a variable degree of cognitive loss. AD is a major public health issue in the US, with an estimated 5.4 million Americans affected by and/or being treated for AD during 2011, at a cost of nearly $183 billion dollars [1]. The exact causes of AD are poorly understood, but a complex etiology is likely, possibly involving multiple genetic, epigenetic, viral and environmental factors [2]. Genetic studies suggest that mutations in the genes encoding presenilin-1, presenilin-2 and the beta-amyloid precursor protein are strong risk factors for AD in the relatively small fraction of AD patients who present with early onset familial disease. However, the vast majority of AD cases are non-familial, sporadic late onset AD, and it is generally thought that genetic predisposition plays little or no role in late onset AD [3-6].

Several studies suggest that DNA damage accumulates in older neurons, and that persistent DNA damage may play a role in late onset AD. For example, DNA strand breaks are 2-fold more abundant in nuclear DNA from cortical neurons in AD brains than in normal control brains [7] and higher levels of the oxidative DNA base modifications 8-oxo-guanine (8-OdG), 8-hydroxyadenine, 5-hydroxycytosine, 5-hydroxyuracil and 4,6-diamino-5-formamidopyrimidine are found in nuclear and mitochondrial DNA in AD brains [8-11]. Oxidative DNA damage is considered a possible early biomarker for AD [12-17] as well as its precursor syndrome, Mild Cognitive Impairment (MCI) [18]. Recent studies also demonstrate impaired oxidative metabolism in fibroblasts from AD patients [19, 20] and protein oxidation, lipid peroxidation, and other markers of oxidative damage are detected in AD brains [21].

Base excision repair (BER), the primary mechanism for repairing oxidative damage to DNA bases [22], is deficient in brains of AD and MCI patients [23-26], which may explain the higher load of oxidative DNA damage in AD tissues. However, other factors may also play a role, including altered patterns of transcription, translation, oxidant defence systems or protein processing, leading to dysfunctional regulatory networks and additional transcriptional defects [2, 27-32].

As discussed above, much evidence supports the hypothesis that oxidative macromolecular damage plays a role in AD. Here, we investigated this idea by comparing the gene expression profile of normal human fibroblasts exposed to oxidative stress with the gene expression profile of sporadic AD-patient derived fibroblast cell lines. For this study, primary human fibroblasts were derived from normal donors age and sex-matched to donors with AD, and the normal fibroblasts were exposed briefly to hydrogen peroxide (H_2O_2) or menadione and analysed 0, 1, 6 or 12 h after the end of treatment. cDNA microarray technology was used to analyze expression of >14,000 genes in the oxidatively-stressed and untreated normal fibroblasts and in untreated AD patient-derived fibroblasts. AD cells were also analyzed by flow cytometry after exposure to a fluorescein-labelled antibody to 8-OdG. The results of these studies support the hypothesis that AD cells experience chronic oxidative stress, which alters gene expression and likely contributes to AD etiology and/or pathology.

Materials and Methods

Cell culture—Primary cell lines were obtained from Coriell Cell Repositories and maintained at 37°C and 5% CO_2. Normal fibroblast cell lines used were AG05448 and AG09857. AD cell lines were: AG05770 and AG07374. All lines were grown in AmnioMAX II Complete media (Invitrogen, CA, USA). U2OS was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin.

Experimental Treatment—Cells were treated with either 25 μM menadione or 100 μM H_2O_2, prepared in media without serum. Treatment with H_2O_2 was carried out for 15 minutes whereas menadione treatment was for 1 h. Post-treatment, the cells were washed once with 10 mL PBS. Conditioned media was returned to the plates then the cells were cultured at 37°C until they were harvested. Cells were harvested after 0, 1, 6, or 12 h. The control cells were manipulated as the experimental except no drug was added (i.e., mock incubation).

Flow Cytometry—Treatment of the cells was carried out as above. Cells were trypsinized and collected in Phosphate buffered saline (PBS) with 10% serum. They were then spun at 400 × g, 4°C for 5 min and washed twice with PBS. Cells were passed through a needle and fixed overnight using 70% ethanol. Fixed cells were washed the next day with PBS. They were incubated in 1 mL PBS with horse serum at 4°C for 30 min. They were further washed in PBS and incubated in 2 N HCl for 20 min. The cells were passed through a needle again and collected by centrifugation as above. Buffer containing 0.1M sodium borate (pH 8.5) was then added for 2 min, followed by centrifugation. The supernatant was collected and incubated with the primary antibody to 8-OdG (Millipore; 1:250) for 2 h at 4°C. The primary antibody was removed by washing 3 times with PBS. The cells were then incubated with a FITC-coupled secondary antibody at 1:1000 dilution for 1 h at 4°C, followed by 3 washes using PBS. The cells were suspended in 1 mL PBS and analysed on an Accuri C6 flow cytometer. The data were analysed using FLOJO software.

Microarray analysis

Total RNA was extracted from normal and AD fibroblasts using QIAGEN RNeasy Mini Kit according to the manufacturer's protocol. The quantity of recovered RNA was assessed using a NanoDrop ND-1000 spectrophotometer. RNA from the 2 cell lines was pooled prior to analysis [33].

A 0.5 μg aliquot of total RNA from the pooled normal or AD cell lines was labeled using the Illumina Total Prep RNA Amplification kit (Ambion). A total of 0.85 μg of biotin-labeled cRNA was hybridized for 16 h to the 23 k gene Illumina's Sentrix HumanRef-8 v2 Expression BeadChips (Illumina). The arrays were washed, blocked and then hybridized. Biotinylated cRNA was detected with streptavidin-Cy3 and quantitated using Illumina's BeadStation 500GX Genetic Analysis Systems scanner. Image processing and data extraction were performed using BeadStudio ver. 15 (Illumina).

Microarray data were analyzed using DIANE 6.0, a spread sheet-based microarray analysis program based on the JMP6 package. Raw microarray data were subjected to Z normalization and tested for significant changes. Genes were determined to be differentially expressed after calculating the Z-ratio and false discovery rate and filtered by ANOVA test and detection values. Individual genes with *P*-value <0.02, Z-ratio >1.5 and false discovery rate <0.03 were considered significantly changed with one way ANOVA by sample p values less or equal to 0.05. Gene set enrichment analysis was used to identify biological and phenotypic changes using the gene expression change of all genes on the array to identify the most significantly changed terms with a Z-test score not more than 0.05 and each selected phenotype term having at least three genes on the array [34, 35]. The functional gene sets used were from the mSIG and Gene Ontology (GO) databases while the disease gene sets were from the GAD database.

Quantitative PCR—Nine genes of interest were chosen based on microarray data, their relevance to AD onset or pathology or their expression level. Total RNA was extracted and used to prepare cDNA with an iScript cDNA synthesis kit (Biorad). RT-PCR using TAQMAN probes (see Table 1) was conducted as per manufacturer's instructions using the Applied Biosystems 7900 HT Real-time PCR system. Each assay was replicated four times and CT values were averaged. Control reactions revealed no significant contamination or cross-contamination of samples.

Results

Experimental Design

Experimental systems for studying AD often use tissue from autopsied brains or peripheral lymphoblasts. In this study, cell lines derived from sporadic AD patient fibroblasts and normal human fibroblasts (controls) from donors age- and sex-matched to the AD patient donors, were used as sources of mRNA for gene expression analysis [36]. The normal cell lines were AG04558, AG09857 and the AD cell lines were AG07374, AG05770. The popoluation doubling and the passage number were the same for the different cells used. Control cells were exposed to 100 μM H_2O_2 for 15 min or 25 μM menadione for 1 h, and

harvested 0, 1, 6, or 12 h after treatment (Figure. S1). Gene expression was also analysed in untreated control cells and untreated AD cells after mock incubations. mRNA was prepared and pooled from two biologically-independent samples for each experimental condition, because this minimized data variability [33]. mRNA was hybridized in triplicate to the 23 k gene Sentrix HumanRef-8 v2 Expression BeadChips (Illumina). Of 14,422 genes analyzed, a total of 1,017 genes (approximately 7%) were up- or down-regulated in AD and oxidatively-stressed normal cells that had a Z-score >1.5 across all the time points measured (see Methods for experimental details and statistical analysis).

Identification of normal fibroblast genes modulated by oxidative stress

Normal human fibroblasts AG04558 and AG09857 were exposed to H_2O_2 (15 min) or menadione (1 h) after which the cells were incubated and harvested for mRNA as described above. When AD cells, relative to untreated normal control cells, were analysed after mock incubations, more genes were down-regulated than up-regulated at both time points (Figures 1A and 1B). At the 0 h time point for the stressed control cells (stressed control versus untreated control, Figure 1A), fewer genes were down-regulated than up-regulated while the reverse was observed at the 12 h time point (Figure 1B). Using a Z-score cut-off of 1.5, 275 or 752 genes were differentially expressed at 0 and 12 h time points, respectively (Figure 1A and 1B). These data suggest that oxidative stress has a greater tendency to down-regulate gene expression than to up-regulate gene expression in normal human fibroblasts.

Common patterns of gene expression associated with oxidative stress and AD

To investigate whether oxidative stress plays a role in AD etiology or pathology, the expression profile of normal cells exposed to hydrogen peroxide or menadione was compared with the expression profile of untreated AD cells; in each case, relative expression values in untreated normal cells served as the reference point. For all four samples/conditions, data were examined at the 0, 1, 6, and 12 h time points after treatment (or mock treatment, for AD cells and untreated normal cells). Data for the 0 and 12 h time points are summarized in Figure 2 and data for the 1 and 6 h time points are summarized in Figure S2. Data for the 0, 1 and 6 h time points were relatively uninformative, because few genes met the cut-off criteria (Z-score > 1.5) and of those, few were up- or down-regulated in both normal cells and AD cells within 6 h after oxidative stress (Figure 2A, S2A and S2B).

In contrast, at the 12 h time point, 752 genes met the criteria for differential expression (i.e., Z-score > 1.5). Of these, 523 genes were differentially-expressed in AD cells, 498 were differentially-expressed in hydrogen peroxide-treated normal cells, and 385 were differentially-expressed in menadione-treated normal cells. These data, and the degree of overlap between the three gene sets, are presented in the Venn diagrams in Figures 2A and 2B. The Venn diagrams illustrate the finding that 215 genes (41%) appear to be similarly co-regulated in H_2O_2 –treated normal cells, menadione -treated normal cells and untreated AD cells (Figure 2B). Cell plots showing gene expression ratios vs gene identity were also used to summarize and analyse these data into gene clusters (Figures 2C, 2D, S2C and S2D), revealing an overall trend similar to the trends described above. The results of this analysis support the idea that AD cells experience chronic oxidative stress, and that this state could influence patterns of gene expression associated with AD.

Gene ontology analysis reveals co-regulation of similar pathways in oxidatively stress normal cells and AD cells

To better understand the global impact of oxidative stress and AD on cellular gene expression networks and cell functions, Gene Set Enrichment Analysis was performed to map the experimentally-determined gene expression profile associated with AD onto specific metabolic pathways and cell functions [37, 38]. In this analysis, the 215 co-differentially-expressed genes (Figure 2B) were clustered according to gene ontology (GO) terms [34, 35], and pathways in which three or more genes were up- or down-regulated were identified. Initial analysis of GO terms corresponding to the 12 h data set is shown in Figures 3A and 3B, with more detailed listing of selected affected pathways shown in Figure 3C. A total of 571 pathways were predicted to be differentially-impacted under all three experimental conditions (Figures 3A and 3B), 337 pathways were predicted to be differentially-impacted in AD cells, 356 in hydrogen peroxide-treated cells and 327 in menadione-treated cells.

The overlap between the three sets of pathways shown in Figure 3A included 141 GO terms/pathways (Figures 3B and S4), many of which have been implicated previously as critical to AD pathology (Figure S4). A subset of these pathways and their Z-scores are shown in Figure 3C. We identified four groups of GO terms/cellular functions that were consistently dysregulated across all three datasets (Figure 3C and S4). The first group of GO terms included up-regulation of translation, ribosomal genes and down-regulation of translation regulation. The second group included metabolism and detoxification of reactive oxygen species (ROS) and up-regulation of cytochrome oxidase, NADH dehydrogenase and glutathione peroxidase. The third group included helicases, ATPase activity and pathways that response to DNA damage. The fourth group of down-regulated GO terms included kinase activity, protein phosphorylation and protein dephosphorylation. Other GO terms associated with altered pathways patterns included forebrain activity (down-regulated) and immune response (up-regulated).

Estimating oxidative DNA damage in AD fibroblasts

To test whether AD cells experience chronic oxidative stress, 8-OdG was quantified in fixed cultured AD and control cells using a commercially available monoclonal antibody against 8-OdG, a fluorescein isothiocyanate (FITC)-labeled secondary antibody and flow cytometry. As control experiments, AD cells were pre-treated with RNase and/or DNase and normal cells were treated with H_2O_2 or menadione (Figures 4A, 4B, S5 and data not shown). The results showed that endogenous 8-OdG was higher in AD cells than in untreated normal fibroblasts (Figures 4C and 4D). Consistent with this, an elevated level of 8-OdG was previously reported in brain and peripheral cells from AD patients [13, 18-20].

Validation of microarray

qPCR experiments were performed to validate the gene expression ratios measured by microarray in this study. For this purpose, the following genes were selected, based on their relevance to AD onset or pathology, their expression level or a literature search: IL-6, CDK6, PTPN12 (Protein phosphatase type 2A complex), TROVE 2 and EIF4G3 (translation factors), DDEF2/ASAP2 (a GTPASE activator), DLC1 (involved in forebrain development)

and ATM. In all cases, the PCR data confirmed the results of the microarray study (Figure 5).

Discussion

The goal of this study was to investigate the possible role of oxidative stress in the pathology of AD. We report that a similar group of genes and pathways are differentially expressed in AD patient-derived fibroblasts and in oxidatively-stressed fibroblasts from age- and sex- matched normal donors relative to AD patient donors. We also provide direct evidence that AD cells experience chronic oxidative stress, as measured by the elevated number of unrepaired 8-OdG lesions in AD fibroblasts grown in culture. The findings directly link oxidative stress to molecular characteristics of AD cells, and therefore generate a putative link to AD etiology and/or pathology.

Several other research groups have used microarray technology to study gene expression in AD cells [2, 39-43]. The present study differs from most previous studies, in that gene expression was analyzed in AD patient-derived fibroblasts. It was previously reported that the gene expression profile of tangle-bearing neurons in AD brains have approximately three-times more down-regulated genes (18%) than up-regulated genes (6%) [40], a result confirmed here when gene expression ratios in AD fibroblasts or oxidatively-stressed normal fibroblasts were compared to gene expression ratios in untreated normal fibroblasts (Figure 1C). Furthermore, the present study confirms earlier studies that suggested possible involvement of specific genes and pathways in AD etiology and/or pathology; thus, the credibility of our study and of previous studies is strengthened. Together, the findings support the idea that oxidative stress influences patterns of gene expression in AD cells, and may play a significant role in AD etiology and/or pathology. Nunomura and co-workers demonstrated that RNA oxidation reflected a snapshot of the oxidative state of the cell since RNA is rapidly turned over [44]. The expression pattern we report in AD cells could be a result of oxidative damage to RNA resulting in the corruption of gene expression. It is interesting that despite extensive literature that exists to support the role of mitochondrial dysfunction in the AD pathogenesis [45-47], we did not observe a change in the expression of genes that regulate mitochondrial function. It is important to note that neither of the two treatments gave a complete overlap in the expression pattern with the AD cells. We speculate that the treatment length, concentration and other factors could have contributed to this and thus to the lack of mitochondrial gene expression changes in our study.

It is also interesting to note the lack of evidence that BER is up-regulated in response to oxidative stress in the context of AD. This calls into question whether expression of BER genes is a valid surrogate marker for oxidative stress [48] or DNA damage, an idea that has been questioned previously, because it is generally recognized that BER genes are constitutively-expressed, and are not induced by DNA damage [49]. However, the basal level at which BER genes are expressed varies with tissue type and with age; so, inadequate BER capacity might still play a role in AD or other aging-related diseases. We believe this question warrants further study.

Finally, we would like to note that in our measurement of 8-OdG lesions in the cultured fibroblasts, we failed to increase this level in the control cells to the endogenous 8-OdG levels found in the AD cells even after treatment with oxidative stress (Figure 4D). This could be one reason for why we do not observe a complete overlap in the expression patterns between control cells treated with oxidative stress and the AD cells The increased endogenous levels of oxidative DNA damage in the AD cells likely is due to the chronic stress exposure that they are under.

Below is a brief discussion of some of the more interesting gene expression findings from our analysis. For some of the genes, there is prior literature on them with respect to AD, whereas others are novel genes that we propose may have a role in the response to oxidative stress in AD cells. In Figure 6, we summarize some of the more interesting genes that we find commonly altered after oxidative stress treatment to normal cells or in untreated AD cells relative to untreated control cells. We used Pubmatrix [50] to identify which of these genes had been previously reported in the literature with AD and those gene products are depicted in red.

IL-6

IL-6 is an inflammatory cytokine secreted by lymphocyte cells during acute infection [51]. Previous reports indicate that IL-6 is involved in the pathology of AD [52-54]. In particular, a polymorphism in the 3′ flanking region of the IL-6 gene is specifically associated with late onset AD and with low risk of AD, results that have been interpreted as evidence that IL-6 expression correlates negatively with AD risk [55], at least in some populations. Interestingly, the same polymorphism has been linked to increased risk of sporadic AD in a different subpopulation of white Americans [56]. In the present study, expression of IL-6 was lower in AD cells and oxidatively-stressed normal fibroblasts (Figure 3B, S3, and Figure 5). Another recent study proposed that IL-6 promotes removal of amyloid-β deposits [57]. Thus, it is possible that IL-6 stimulates clearing of deleterious AD-associated plaques by microglia, explaining the antagonism between IL-6 and AD risk. Future studies of the effect of oxidative stress on expression of IL-6 might be interesting, and it may be premature to rule out the possibility that IL-6 could be a useful therapeutic target for AD.

ATM

Ataxia-Telangiectasia (A-T) is a progressive cerebellar ataxia characterized by neurological manifestations, conjunctival and cutaneous telangiectasia and recurrent sino-pulmonary infections [58]. The gene mutated in A-T encodes a kinase, ATM, which plays an important role in the DNA damage signaling cascade (DDR) [59]. Furthermore, lack of ATM is associated with high levels of ROS, which in turn activates the ATM-dependent DDR in oxidatively-stressed ATM-proficient cells [60, 61]. Interestingly, *ATM-/-* mice have an abnormally high number of lysosomes, fueling speculation that A-T and AD are in some way related or similar [62]. The present study shows that ATM mRNA is less abundant in AD fibroblasts and oxidatively-stressed fibroblasts than in untreated normal fibroblasts. However, additional studies are needed to confirm these results and further to determine the significance of this observation.

PRKAA1

Mammalian AMP-activated protein kinase, AMPK, is a heterotrimeric complex containing a catalytic subunit, αl or α2, and two regulatory subunits, β and γ. PRKAA1 and PRKAA1 code for the αl and α2 subunits of AMPK. AMPKα1 is expressed in multiple tissues like brain, heart, liver, kidney etc, whereas AMPKα2 is more abundant in skeletal muscle and heart and less so in liver and kidney [63]. AMPK plays a major role in cellular energy homeostasis by regulating lipid and glucose metabolism in response to AMP levels and other stressors [64]. AMPK is activated after DNA damage by ionizing radiation [65], regulates ROS, autophagy, cell proliferation, and mitochondrial functions [64]. In AD, AMPK has been reported to be over activated and it directly regulates tau phosphorylation [66]. Here we report loss of PRKAA1 mRNA after oxidative stress and in AD fibroblasts. As a result, AD cells may not have a normal DNA damage response and additionally would need to preferentially rely upon PRKAA2, which may have altered tissue expression patterns as well as other catalytic differences.

BDNF

Brain-derived neurotrophic factor, BDNF, is a widely expressed brain growth factor that plays important roles in neuronal viability, synaptic plasticity, neurogenesis and aging [67, 68]. Mixed findings have been reported for the association of AD and BDNF levels. Some reports state that BDNF levels are elevated in AD [69, 70] whereas others have reported lower BDNF levels in AD patients [69-73]. Here, we report that BDNF mRNA is down-regulated after exogenous oxidative stress. Therefore, our results suggest that BDNF expression goes down after oxidative stress and support the hypothesis that loss of BDNF, in response to oxidative stress, may make neurons more sensitive to oxidant-induced cell death.

NEDD4

NEDD4 (neural-precursor cell-expressed developmentally down-regulated gene 4) is a HECT(homology to E6-AP carboxy terminus) E3 ubiquitin ligase, which shows high expression in the embryonic mouse CNS [74]. E3 ligases are involved in the ubiquitination of several neuronal proteins, which controls their endocytosis and endocytic sorting [75-79]. It is thought that the endocytic-lysosomal pathway plays an important role in neurodegeneration and protein aggregation is a hallmark of AD [80]. After DNA damage that blocks transcription, NEDD4 is known to bind to and ubiquitinate RNA polymerase II, which is necessary for DNA repair [81]. In our study, we find a dramatic down-regulation of the NEDD4 gene transcription in the AD samples and in the control cells when treated with oxidative stress. This in turn could lead lysosomal dysfunction, leading to protein aggregation in AD, and could also lead to aberrant DNA repair of transcription blocking lesions.

ZAK

The zipper sterile-a-motif kinase (ZAK) is a serine/threonine kinase that activates the c-Jun amino-terminal kinase (JNK) and the mitogen-activated-protein kinase (MAPK p38) pathways [82]. These kinases respond to a variety of stressors and some cellular endpoints

include apoptosis, cell cycle, and metabolism. It has been reported that transfection of a kinase-dead ZAK leads to disruption of actin stress fibers [83]. Another function of ZAK is to arrest cell proliferation by causing a G2 cell cycle arrest [83]. Specifically, it has been shown that ZAK participates in the G2 arrest of cells after DNA damage by gamma radiation [84]. In our study, we observed significant down-regulation of ZAK in AD and normal human fibroblasts after oxidative stress. There is no literature on ZAK and AD but loss of ZAK may compromise the ability of AD neurons to induce an appropriate cell cycle checkpoint after damage.

ZMAT3

ZMAT3 or Wig1 was discovered as a p53 transcriptional target [85, 86]. Later studies demonstrated the ability of ZMAT3 to bind to double stranded RNA, besides DNA, and is thought to play a role similar to that of miRNA [87-89]. Interestingly, its expression was found to be highest in the brain [89]. Furthermore, ZMAT3's expression was induced in the nervous system by a number of stress agents, including ischemia, treatment with methamphetamine, onset of disease in a model of ALS, and by L-DOPA in a Parkinson model [90-94]. These data indicate a role for ZMAT3 in cellular stress responses and pathological conditions in the nervous system. ZMAT expression was down-regulated in our study, suggesting a role for this protein in the oxidative stress response in both normal and AD cells.

Top2B

Topoisomerase IIβ is a type II topoisomerase, an enzyme involved in cutting and resealing double stranded DNA, and important for DNA replication and transcription [95]. Top2B is expressed in post-mitotic cells, implying its importance in the maintenance of tissue homeostasis [96-98]. A previous report suggested a role for this enzyme in neural cell differentiation and another reported aberrant lamination in the cerebral cortex of mouse embryos lacking DNA Top2B [99, 100]. Our microarray analysis revealed a down-regulation of Top2B transcripts; whether it plays a role in the response to oxidative stress in AD cells will need additional research.

Lastly, AD is a complex disease with many cellular pathways and proteins deregulated. How all these events contribute to the pathology observed in AD warrants intensive research efforts. AD is the scourge of our generation and its toll on society will only increase with time. Here we have identified oxidative-stress responsive mRNAs of previously accepted AD-associated genes and novel genes that may play a role in the complex pathology of AD. In the future, we hope to further characterize if some of the altered genes identified here show alterations in primary AD tissues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Alzheimers fibroblasts (AD) have an oxidative stress phenotype
- AD fibroblasts have increased 8-oxoG in their DNA
- Many genes and pathways common to AD fibroblasts and stressed normal fibroblasts

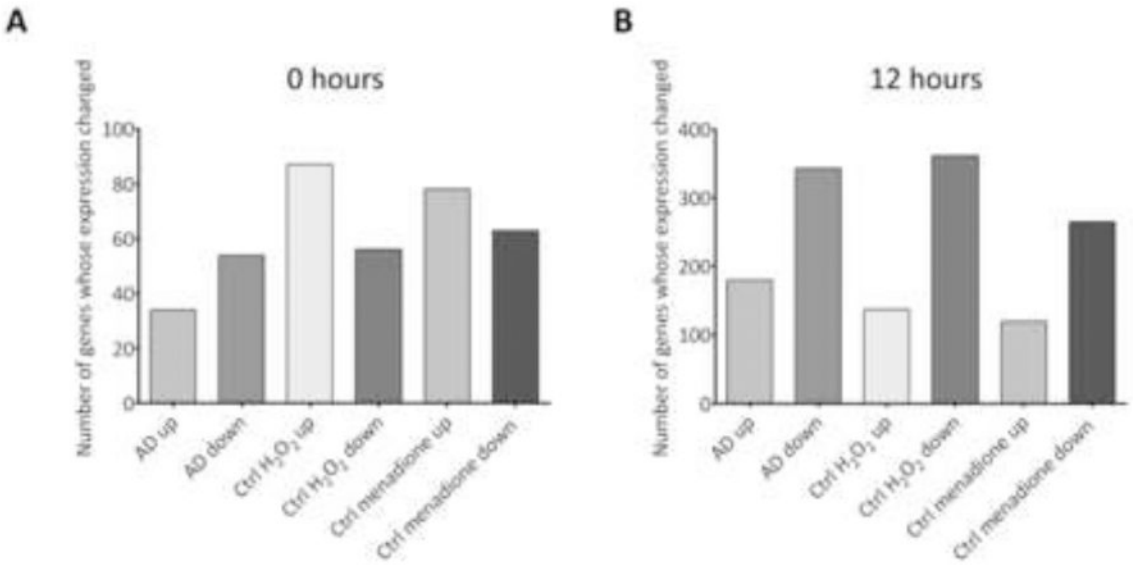
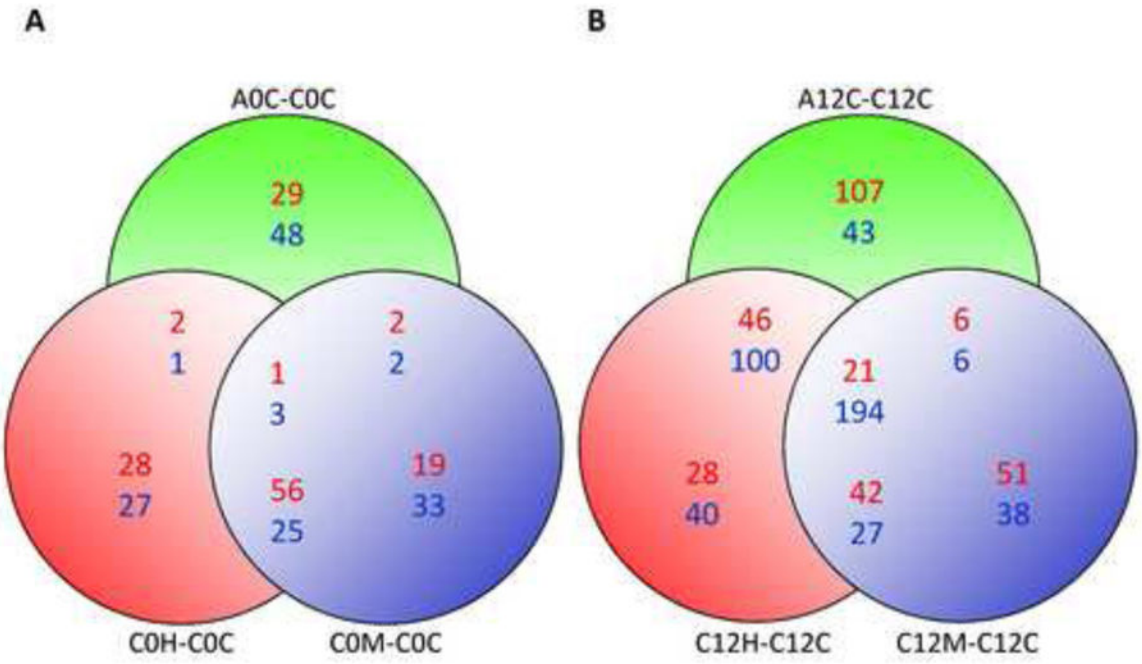


Figure 1. The response of normal human fibroblasts to oxidative stress

(**A**) Bar graphs plotting number of genes up- (up) or down-regulated (down) 0 h after treatment with 100 μM H_2O_2, or 25 μM menadione (normal fibroblasts) or after mock incubation (AD cells and control cells) (**B**) Same as (A), except 12 hours after treatment or mock treatment.



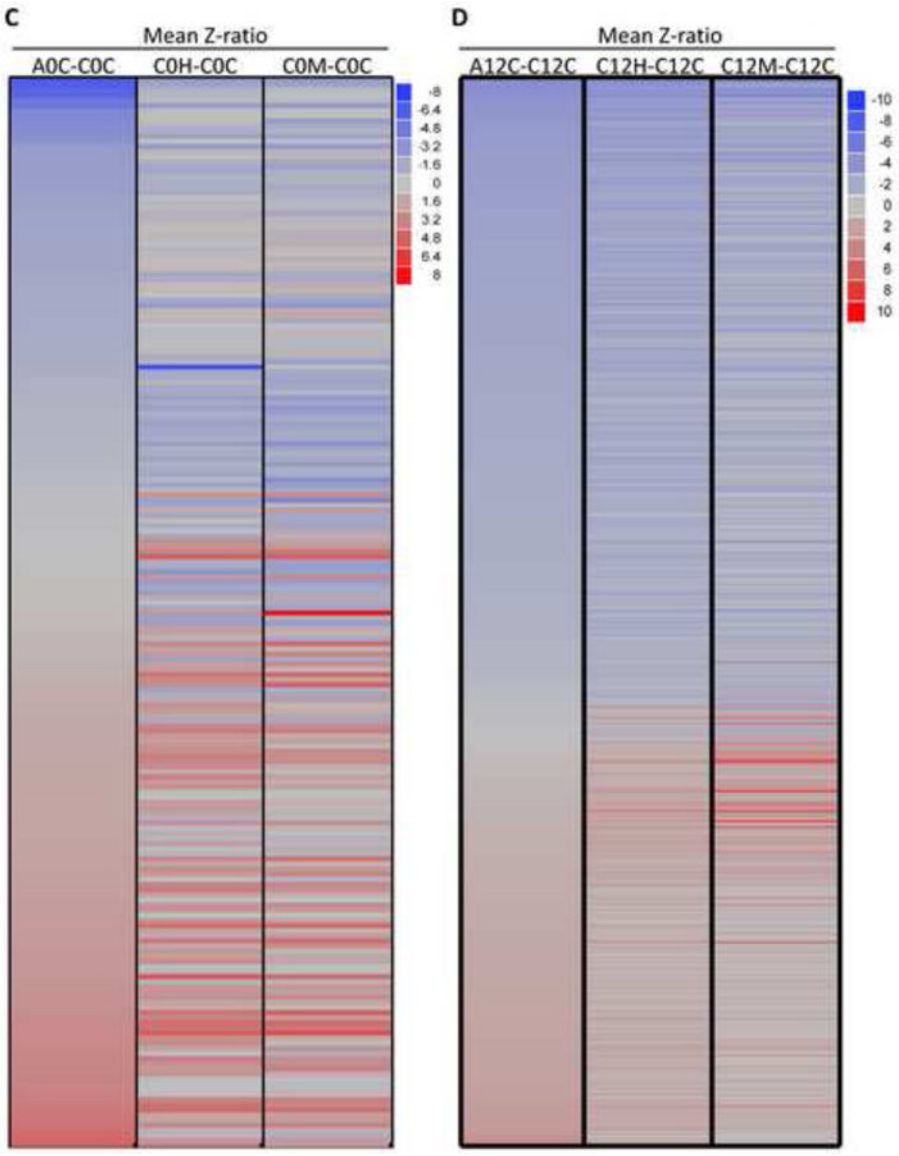
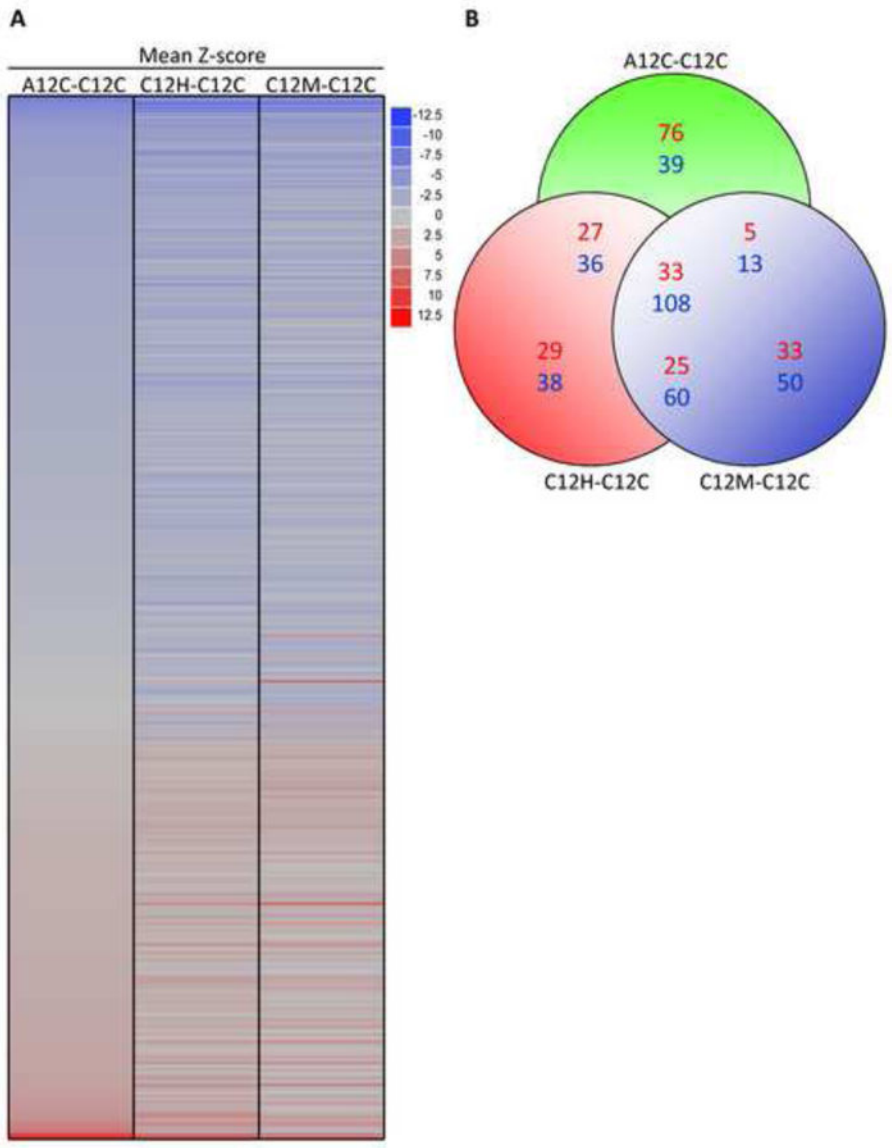


Figure 2. Gene expression profiles associated with AD or oxidative stress

(A) Venn diagram showing the number of genes co-regulated in unstressed AD cells (A0C-C0C); H_2O_2 treated normal fibroblasts (C0H-C0C) or menadione-treated normal fibroblasts (C0M-COC) using cells harvested immediately after the end of treatment or mock treatment. Up-regulated genes are shown in red and down-regulated genes are shown in blue. (B) Same as (A), except using cells harvested 12 h after the end of treatment or mock treatment. (C) Heat map/Cell plot summarizing gene expression ratios immediately after the end of treatment or mock treatment. The color key on the right maps color intensity to the range of positive and negative expression ratios. (D) Same as (C), except using cells harvested 12 h after the end of treatment or mock treatment.



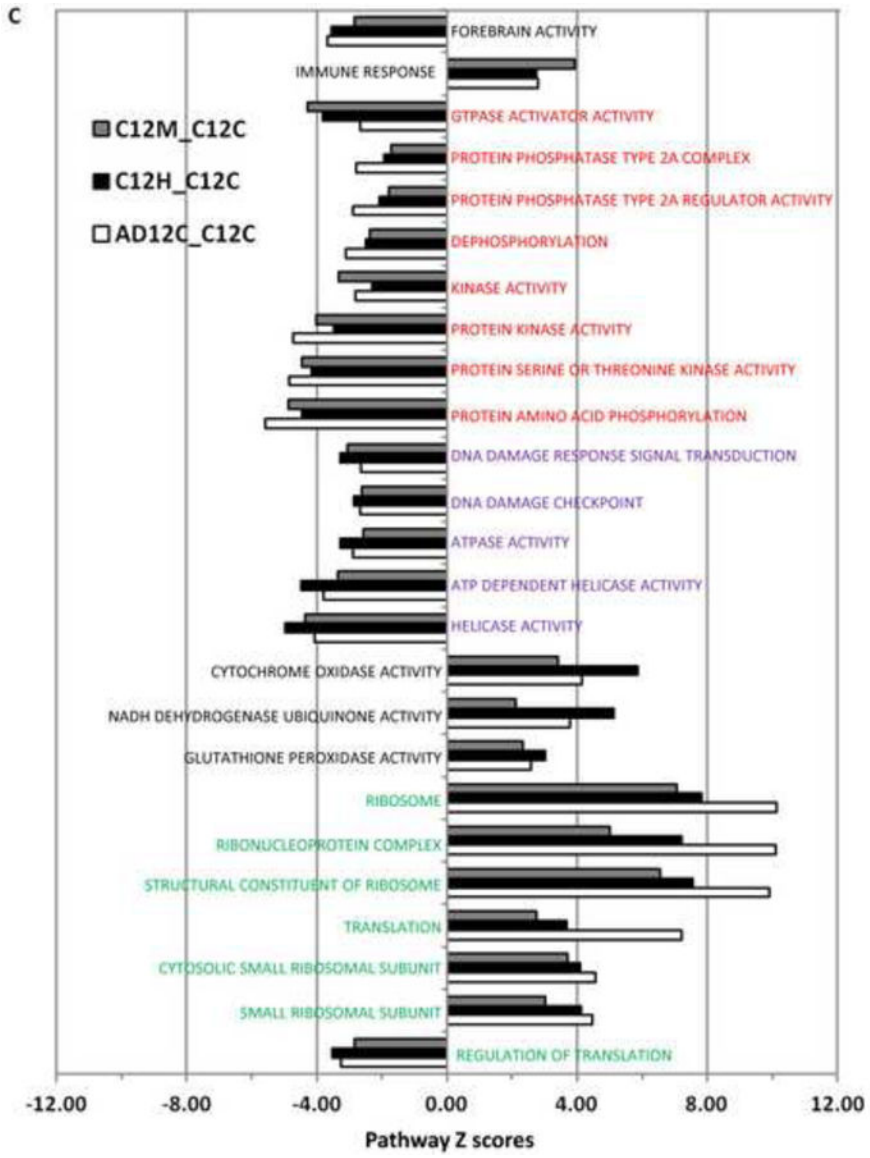


Figure 3. Oxidative stress triggers overlap of gene ontology changes that resemble Alzheimer's disease expression profile

(A) Heat map summarizing GO terms linked to significant gene expression ratios shown in Figure 2A (see text for details). (B) Venn diagram number of GO-terms/pathways co-regulated in unstressed AD cells (A12C-C12C); H_2O_2 treated normal fibroblasts (C12H-C2C) or menadione-treated normal fibroblasts (C12M-C2C) using cells harvested 12 h after the end of treatment or mock treatment. The color key on the right maps color intensity to the range of positive and negative expression ratios. (C) Selected prominent GO terms/ pathways from (A), with corresponding Z-scores, thought to have potential relevance to AD.

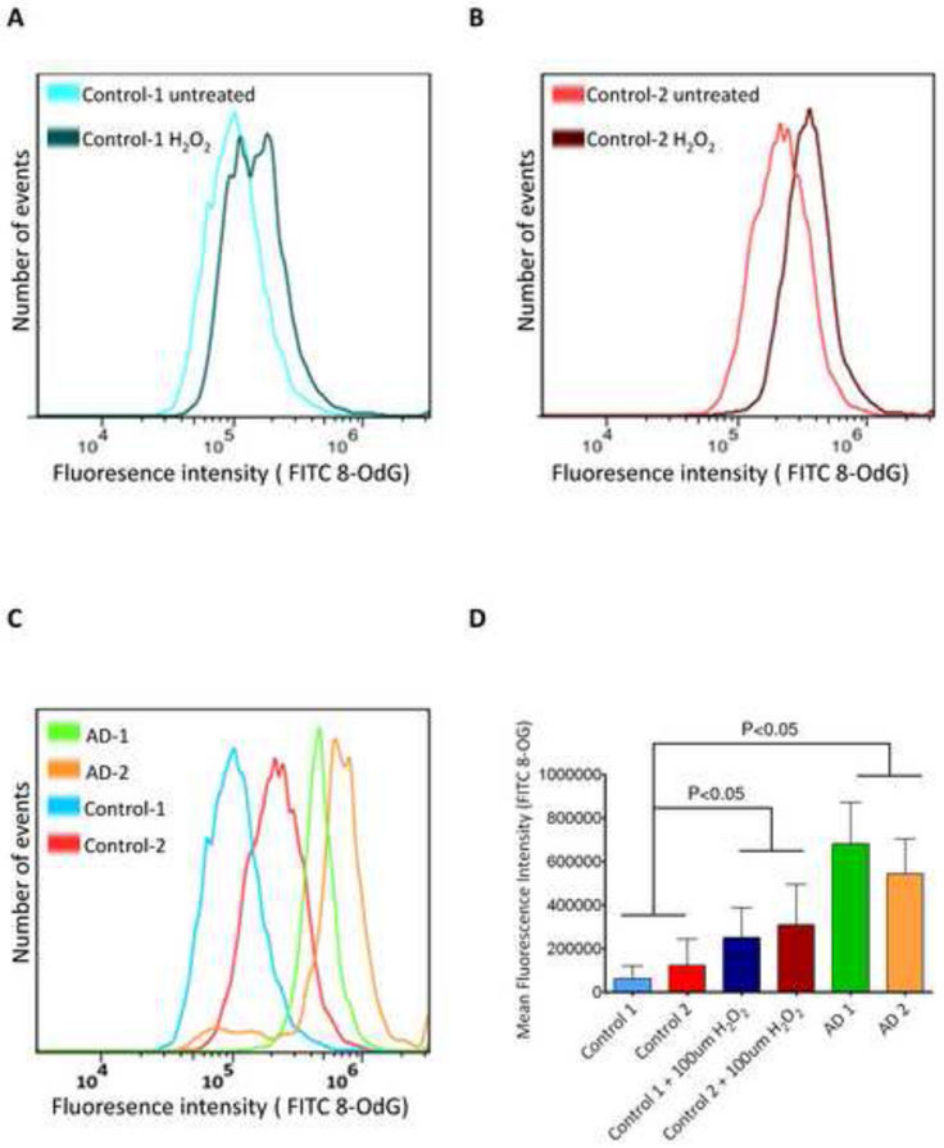


Figure 4. Flow cytometric quantitative analysis of 8-OdG in AD fibroblasts grown in culture

(**A**) Quantified FITC intensity as indicator of 8-OdG in fixed normal fibroblasts (control 1) after exposure to 100 μM H_2O_2 for 15 min. (**B**) As in (A), except using untreated normal fibroblasts (control 1). (**C**) As in (A), except using untreated normal fibroblasts (control 1), untreated normal fibroblasts (control 2), untreated AD fibroblasts (AG07374) and untreated AD fibroblasts (AG05770). (**D**) Quantification of the data shown in (C) using the mean value across different cell lines. Error bars show ±SEM, n=2 per cell line.

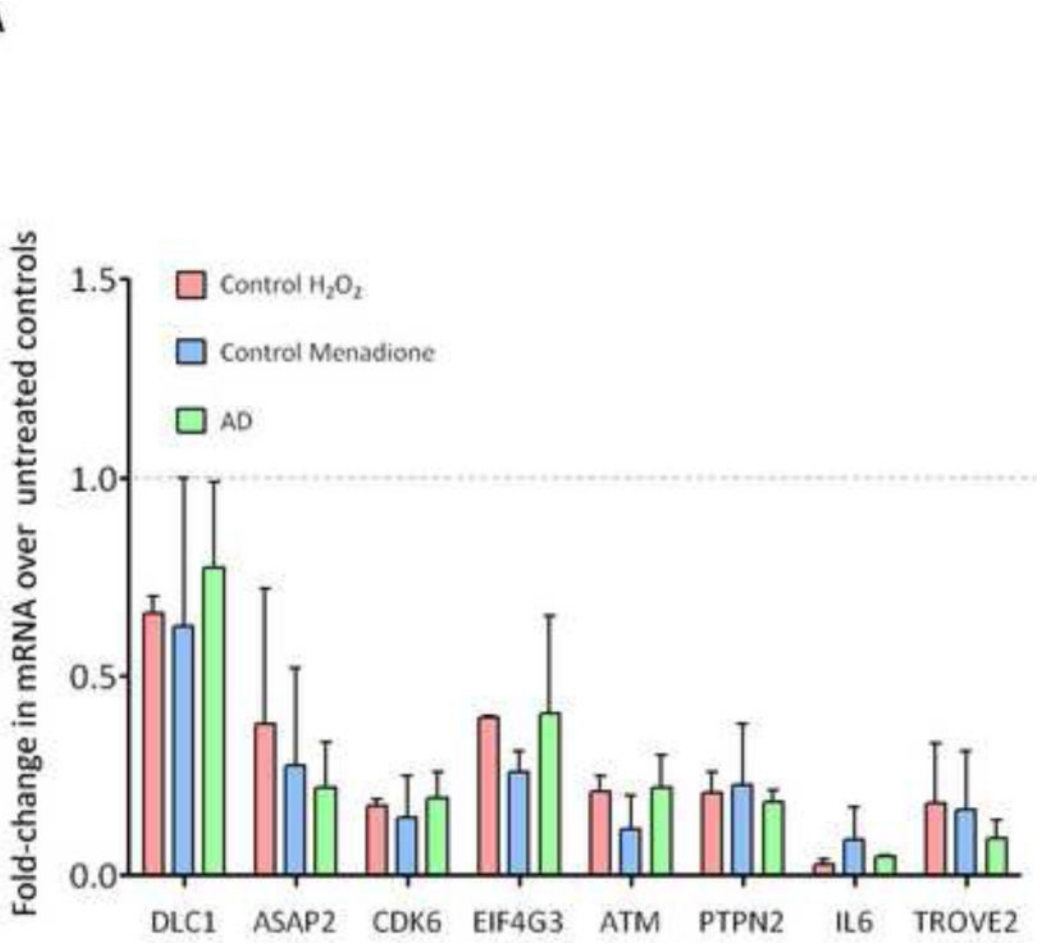


Figure 5. Validation of microarray data

Fold-change in expression of the indicated genes was determined using quantitative PCR. Error bars show ±SD, n=3

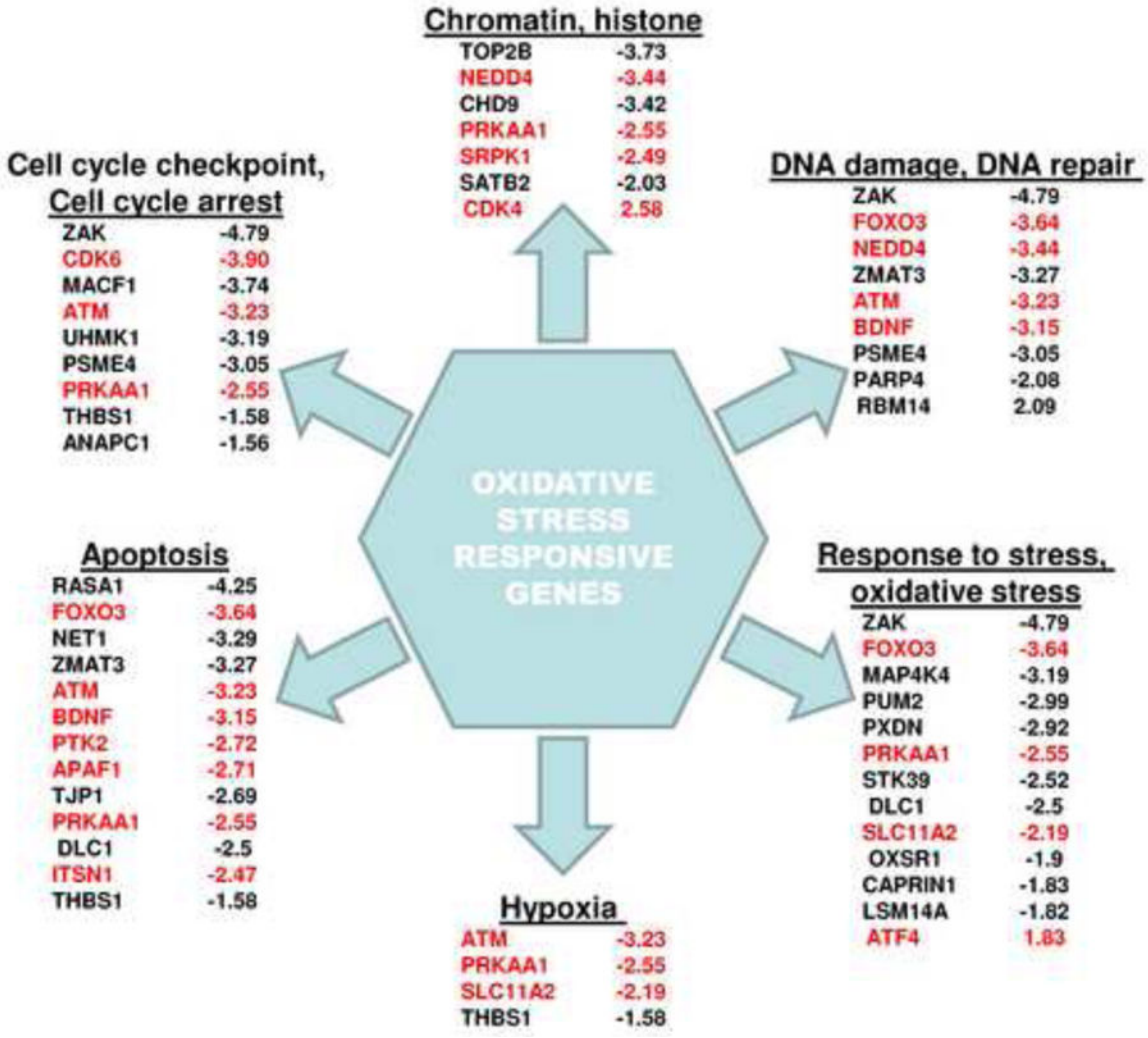


Figure 6. Summary of Pubmatrix search

Summary of some of the more interesting genes that we find commonly altered after oxidative stress treatment to normal cells or in untreated AD cells relative to untreated control cells. Pubmatrix [50] was used to identify which of these genes had been previously reported in the literature with AD and those gene products are depicted in red.