수의학석사 학위논문

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2017년 2월

서울대학교 대학원

수의학과 수의미생물학 전공

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A thesis submitted to the faculty of the
Graduate School of Seoul National University
in partial fulfillment of the requirement for
the degree of Master in Veterinary Medicine

February 2017

Department of Veterinary Medicine
(Major: Veterinary Microbiology)
The Graduate School

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지도교수 우 희 종

이 논문을 수의학석사 학위논문으로 제출함
2016 년 12 월

서울대학교 대학원
수의학과 수의미생물학 전공

김 민 선

김민선의 수의학석사 학위논문을 인준함
2016 년 12 월

위 원 장: 유 한 상 (인)
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Abstract

Differentially expressed genes in prion protein conversion induced by trivalent iron

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The conversion of the cellular prion protein (PrP^{c}) to protease-resistant isoform is the key event in chronic neurodegenerative diseases including transmissible spongiform encephalopathies (TSEs). Increase of iron in prion related disease has been observed due to the prion protein-ferritin complex. The accumulation and conversion of recombinant PrP (rPrP) was specifically derived by Fe(III) but not by Fe(II). Fe(III)-mediated PK-resistant PrP (PrP^{res}) conversion was conducted with a more complex cellular environment instead of a direct contact between rPrP and Fe(III). In this study, the differentially

expressed genes that correlate with the prion degeneration by Fe(III) were identified using Affymetrix microarrays. In Fe(III)-treated environment, 97 genes were differentially expressed, 85 upregulated and 12 downregulated ($\geq$1.5-fold change in expression). However, Fe(II)-treated environment produced moderately altered gene expression level and did not induce a dramatic alteration of gene expression profile. Moreover, functional grouping of identified genes indicated that the differentially regulated genes were highly associated with cell growth and maintenance, intra and extracellular transport environments. These findings show that Fe(III) might influence the expression of genes for PrP folding by redox mechanism. The identification of genes with altered expression patterns in neural cells may provide insight to the PrP conversion mechanisms in development and progression of prion related diseases.

Keywords: iron, conversion, prion disease, microarray, gene expression, redox

Student Number: 2012-21536

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INTRODUCTION

Fatal neurodegenerative prion disorders result from protein misfolding [1]. Prion protein is the most fundamental substance in prion diseases, or transmissible spongiform encephalopathies (TSEs) including scrapie in sheep, chronic wasting disease (CWD) in elk and deer, bovine spongiform encephalopathy (BSE) in cattle and variant Creutzfeldt-Jakob disease (vCJD) in human. During disease, a conformational change of normal prion protein (PrP^{c}) to disease specific PrP isoform (PrP^{Sc}) causes protease digestion resistant and water insolubility that renders the resultant protease resistant PrP (PrP^{res}). In several functional roles of PrP^{c}, a relationship between PrP^{c} and metal homeostasis especially of iron and copper has been proposed [2, 3]. Oxidative stress including redox process have been observed because of the imbalance of metal homeostasis in diseased brain [4, 5]. Two oxidative states of iron are believed to implicate in prion diseases [4, 6-8]. In our previous study, the conversion and intracellular accumulation of recombinant PrP (rPrP) were specifically derived by Fe(III) rather than Fe(II). Furthermore, Fe(III) did not induce PrP^{res} formation in a direct contact with rPrP. Fe(III)-mediated rPrP conversion to PrP^{res} requires a complex cellular environment [9]. Though the pathogenic mechanisms of neurodegeneration especially the generation of the

infectious isoform of PrP (PrP^{Sc}) have been studied in many ways [3, 5, 10, 11], factors for the acquisition of protease resistance are not completely defined. As a useful technique, microarray analysis has performed to obtain information of the factors involved in disease development and progressing and several studies have been conducted to identify gene expression changes related with prion disease [12-14]. In this study, we identified differentially expressed genes correlated with prion degeneration depend on oxidative states of iron using Affymetrix microarray and total RNA samples extracted from cells treated with iron and rPrP. Gene Ontology (GO) annotations was performed to indicate the functional grouping of differentially regulated genes of either down or upregulated.

MATERIALS AND METHODS

1. Generation of recombinant protein

Bovine rPrP was cloned in a pET23 vector (Novagen, Madison, WI, USA) and expressed in *Escherichia coli* BL21 (DE3). Induction was performed with 1 mM isopropyl β-D-1-thiogalactopyranosid (IPTG) for 16 h before cloning. Cells were resuspended and sonicated in cold phosphate-buffered saline (PBS) containing protease inhibitor cocktail and 5 mM ethylenediaminetetraacetic acid (EDTA). Samples were centrifuged at 50,000 × g for 30 min; solubilized with 20 mM sodium phosphate (pH 7.4), 0.5 M NaCl, 20 mM imidazole, and 6 M guanidine-HCl; and then sonicated briefly. The solubilized proteins were collected again by centrifugation at 50,000 × g for 30 min. A HisTrap HP column (GE Healthcare, WI, USA) was used for elution of cleared lysates with 20 mM sodium phosphate, (pH 7.4), 0.5 M NaCl, 0.5 M imidazole, and 6 M guanidine-HCl. Eluted rPrP was dialyzed against 50 mM sodium acetate (pH 5.0) containing 5% glycerol and then redialyzed against 20 mM HEPES (pH 7.4).

2. Cell culture and treatment with iron and rPrP

HpL3-4 cells from the hippocampus of PrP-deficient mice [15] were seeded at a density of 0.4×10^5 cells/mL in the high glucose Dulbecco's modified Eagle's

medium (DMEM) containing heat-inactivated 10% fetal bovine serum (FBS) and maintained at 37℃ with 5% CO_2. After 4 h of cell stabilization, a range of concentrations of either ferrous chloride ($FeCl_2$) [Fe(II)] or ferric ammonium citrate (FAC) [Fe(III)] was added to HpL3-4 cultures for 24 h. Subsequently, cells were exposed to 0.6 μM rPrP for an additional 24 h. Mock-exposed cells treated only with rPrP were used as a control.

3. Whole cell lysates preparation

After trypsinization, cells were collected and washed with PBS five times to exclude contamination with rPrP from media. Cells were then lysed in lysis buffer containing protease inhibitor cocktail, 2% NP-40, 1% sodium deoxycholate, 20 mM Tris-HCl, 150 mM NaCl, 0.25% sodium dodecyl sulfate (SDS), and 2 mM EDTA for 30 min at 4℃. After centrifugation at 1200 rpm for 5 min, the supernatant was collected.

4. Proteinase K treatment

After iron and rPrP treatment, the PBS-washed cells were lysed in lysis buffer containing 2% NP-40, 1% sodium deoxycholate, 20 mM Tris-HCl, 150 mM NaCl, 0.25% sodium dodecyl sulfate (SDS), and 2 mM EDTA for 30 min at 4℃. For the protein digest reaction, 1 or 5 μg/mL PK was added in 1 mg/mL of each

protein sample at 37℃ for 30 min. PK digestion was stopped by the addition of 2 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentrations were measured using BSA Protein Assay Reagents (Fisher Scientific, MA, USA).

5. Western blot analysis

Western blot analysis was performed to confirm accumulation of internalized PrP and other proteins. Sample proteins subjected to 15% sodium dodesyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto polyvinylidene difuoride (PVDF) membranes (Millipore; Immobilon-P membranes). The membranes were blocked using 5% nonfat dry milk in TBS-Tween for 2 h at room temperature. Membranes were probed overnight by using anti-PrP antibodies (1E4; 1:5000 dilution) and β-actin antibodies (1:5000 dilution) as primary antibodies, and appropriate secondary antibodies were applied. The blots were developed by enhanced chemiluminescence (Vilber, Fusion SL), and the relative intensity of the proteins was analyzed using Fusion Software.

6. RNA isolation and purification

Cells were collected and washed with PBS five times. Total RNA from each sample of cells was extracted and purified using an RNeasy mini kit (Qiagen,

Valencia, CA, USA) according to the manufacturer's instructions. The quality of total RNA samples was evaluated using an Agilent 2100 bioanalyzer (Agilent Technologies, CA, USA) and agarose gel electrophoresis to ensure equal purity and the absence of degradation for all samples. Total RNA samples were quantified using an ND-1000 Nanodrop analyzer.

7. Microarray, statistical analyses, and data mining

According to the labeling protocol developed by Affymetrix (Santa Clara, CA, USA), 10 μg of each total RNA sample was labeled. Biotin-labeled cRNA was hybridized on Affymetrix GeneChip Mouse 2.0 ST Arrays, which contained 35,240 probe sets corresponding to ~26,191 well-characterized mouse genes. After hybridization, the arrays were stained, washed using a GeneChip Fluidics Station 450, and scanned using a GeneChip Array Scanner 3000 7G. Data were extracted using Affymetrix Expression Console1.3.1, and probe detection calls and cell intensity values were then computed. For array data normalization, Robust Multiarray Average Normalization was applied. Probe sets with adjusted intensity values of greater than 100 in at least one of the arrays were used as filtered data. P values were adjusted in order to control the false discovery rates (FDRs) of multiple comparisons using Benjamini and Hochberg's method [16]. Genes with fold change value of greater than 1.5 among samples (upregulated or

downregulated) were considered differentially expressed. Genes with adjusted P values less than 0.05 were considered differentially expressed for functional grouping and then classified functionally using DAVID Bioinformatics Resources 6.7 [17]. To verify the extracted data, diagnostic plots, such as correlation plots, density and box plots, M&A plots, and visual inspection of array images, were applied according to the recommendations of Affymetrix Microarray Analysis Suite 5.0.

RESULTS

1. Confirmation of PK-resistant internalized rPrP

The presence of the disease specific isoform of the prion protein in cells that treated with two different oxidative states of iron ($FeCl_2$ and FAC) and rPrP was confirmed by Western blot (Fig. 1). Cells were exposed to 0.3, 0.4 and 0.5 mM of either $FeCl_2$ or FAC. Iron concentration dependent accumulation of internalized rPrP was observed in cells treated with FAC, whereas no signal was detected from the mock-exposed control and the insignificant level of internalized rPrP was shown in cells with any concentration of $FeCl_2$ exposure.

Resistance of intracellular rPrP against PK digestion was examined with PK concentrations of 1 and 5 μg/mL after cells were exposed to 0.3, 0.4 and 0.5 mM of FAC (Fig. 2). PK concentration dependent digestion was shown in the same condition of FAC concentration. Partial PK resistance was observed in intact rPrP (25 kDa), yielding a PK-resistant fragment (17kDa) which is designated as PrP^{res}. Even with a minimal FAC concentration of 0.3 mM, rPrP showed PK resistance in 1 μg/mL of PK treatment. 0.3mM of FAC was selected as the optimal concentration of Fe(III) exposure.

To compare with Fe(II) exposed cells, cells were treated with 0.3 mM of either $FeCl_2$ or FAC and then exposed to 1 μg/mL of PK which is minimal PK

concentration (Fig. 3). With PK treatment, rPrP from $FeCl_2$ exposed cells and the control was clearly digested, whereas a high level of internalized rPrP was detected in FAC exposed cells.

2. Microarray analysis

Microarray analysis was performed to identify differentially regulated genes using total RNA obtained from cells treated with $FeCl_2$ and FAC. Evident gene expression changes were observed from Fe(III)-treated environment. 97 genes that were differentially expressed ($\geq$1.5-fold change in expression) were detected. Of these, 85 were upregulated and 12 were downregulated as compared with negative controls. However, virtually moderate changes in the gene expression level were observed at Fe(II)-treated environment from negative control and did not induce a dramatic alteration of gene expression profile. As expected with this result, the number of upregulated genes from Fe(III) exposed samples compared with Fe(II) exposed samples was significantly higher than upregulated genes from Fe(II)-treated samples.

Among the differentially expressed genes, the comparison analysis of the different samples revealed significant and specific genes that regulated differentially. By merging the three comparison data, only 30 genes were filtered

as significantly altered in samples treated with FAC versus either negative control or $FeCl_2$ exposed samples (Fig. 4).

This set of genes was also used as the input for the hierarchical cluster (Fig. 5). As shown in the hierarchical cluster, it is notable that differential gene expression showed consistent patterns when negative control and iron-treated samples were compared.

To assess the functional groups of the selected genes, DAVID Bioinformatics Resources was applied. The majority of identified transcripts have not been classified, however, the patterns of upregulated genes in FAC-exposed samples could be divide into 7 categories according to their Gene Ontology (GO) annotations (Table 1). Cellular trafficking related mechanisms were activated in FAC-treated group (*Amph, Slc40a1, Slpi, Mup5, Adm, Dpt, Rsad2*). Some of genes involved in immune response identified in this study are *Amph, Ly6f, Ly6c1, Ly6c2, Slc40a1, Rsad2*. Another group of genes are those involved in cell death and cell proliferation (*Gzme, Gzmd, Slpi, Adm, Dpt*). Genes such as *Amph, Slc40a1, Rsad2* specifically involved in multiple functional groups indicated that some genes were able to have more than one molecular function. Two of the genes that showed downregulated expression in FAC-treated samples were *S100a7a* and *Tfrc* (Table 2). Both of them were related to metal ion transport.

Based on those results, categorized genes and their associated processes could be related to or play important roles in rPrP degeneration and PrP^{res} accumulation.

DISCUSSION

To identify genes for factors related to the accumulation and conversion of rPrP, we identified 97 genes that were differentially expressed in FAC exposed cells using Affymetrix GeneChip Mouse 2.0 ST Arrays. 30 genes were specifically selected as differentially regulated genes.

Neuronal degeneration and cell death are specific processes of diseased brain [11, 18, 19]. *Gzme, Gzmd and Slpi* genes for cell death and *Adm* and *Dpt* genes for cell proliferation were detected. The observed expression pattern of genes associated with cell death and proliferation indicates a cellular effort to balance between the survival and death of the neuronal cell populations.

In previous study on acquisition of the PK resistance of rPrP, vesicular trafficking was the critical process for Fe(III)-mediated PrP conversion [9, 20, 21]. The existence of factors in vesicles and trafficking would be required for the internalized rPrP to acquire PK resistance because intracellular components within vesicles dynamically transit through homo- and heterotypic membrane fusion along the pathway leading to lysosomes [38]. This suggests that vesicular trafficking may play a role in PrP^{res} formation [39]. Not surprisingly, genes of vesicle-mediated transport such as *Amph* and *Slc40a1* were differentially

upregulated. *Amph, Ly6f, Ly6c1, Ly6c2, Rsad2* and *Slpi* were overtly immune-related genes.

Our results showed an evidence of an activated metal ion binding genes such as *Slc40a1* and *Rsad2* were related in the conversion of rPrP. Slc40a1 known as iron-regulated transporter and *Rsad2* have been observed not only in immune-related group but also in metal ion binding group. Also genes associated with extracellular region were upregulated. These are *Slpi, Mup5, Adm* and *Dpt*. Notably, effects of *Adm* include upregulating angiogenesis and increasing the tolerance of cells to oxidative stress [22]. There is also significant upregulation of a groups of genes involved in cell signaling.

A mild alteration of the downregulated gene expression was detected in FAC exposed cells. Iron accumulation in prion disease has been observed due to PrP-ferritin complex causing iron bio-insufficiency. During conditions of iron deficiency, TfR (Transferrin receptor) levels that is likely to reflect iron saturated Tf levels are upregulated to increase iron uptake [8]. Though an upregulated expression of Tfrc was expected in the FAC-treated cells, it was downregulated drastically. It could be explained by the characteristic degradation of TfR [23], additional studies are needed to support our observation. Alterations in genes on calcium homeostasis are important factors in the degeneration of neurons, as maintenance of calcium homeostasis is critical for neuron survival [24].

Additional evidence of this was provided by the downregulation of *S100a7a* in this study.

Assessed the biological relevance of the data gained in the pathogenesis of prion disease, in contrast to comparable studies of diseased brain [12-14, 25-28], marginal number of the upregulated genes observed. As our results have been limited to *in vitro* experiments with rPrP and the excessive amount of iron, additional factors could be existing. Gene expression could be differing in time after infection using *in vivo* at which altered expression was observed in diseased brain. Though it was required to be confirmed *in vivo*, our study could provide information to clarify the role of Fe(III) in conversion and accumulation of the internalized PrP. Moreover, Quantitative real-time PCR (qRT-PCR) is a commonly used to validate gene expression results obtained from microarray analysis. Therefore, the assessment of analyzing transcript levels for genes significantly changed in each direction or comparison such as *Amph, Slc40al, Adm, S100a7a* and *Tfrc* can provide insight into the potential roles of these genes.

It may be interesting to apply our findings to other neurodegenerative diseases including Parkinson's disease (PD) and Alzheimer's disease (AD). Notably, the pathology of prion disease can include amyloid plaques, similar to those appearing in patient with AD [29]. Reduction of *Amph* which is immune-related gene was validated as a molecular event in AD [30]. *Slc40al* was also found to

have a role in AD pathophysiology [31]. Increased reactive carbonyls lead to oxidative stress which play a significant role in the pathogenesis of neurodegeneration in brains [32]. Upregulation of *Cbr2* and *Rsad2* imply that other neuronal diseases could be influenced by Fe(III)-exposed like environment.

The role of autophagy in prion disease was proposed that autophagy may contribute to conformational change of PrP^{c} to PrP^{Sc}. However, autophage also has a beneficial effect of protecting against neurodegeneration as a defense mechanism [40]. Thus, it is reasonable to assume that genes we found of vesicle-mediated transport such as *Amph* and *Slc40a1* are could be the key factors to understand interconnection between neurodegeneration diseases and cellular pathway of autophagy.

Many studies have shown that elevated ferric iron concentrations promotes the aggregation of contributory proteins in neurodegenerative disorders. The relationship between iron and TF in diseased brains was evaluated by assessment of the TF/iron ratio [33]. This study reflected a tendency of TF concentrations on AD and PD brains to remain the same or decrease, whereas iron increased. Aberrant metabolism or imbalance of iron are a prominent feature of diseased brains [34-36]. As PrP^{c} triggers cellular iron uptake by providing the ferrireductase activity [35, 37], further correlations between prion and other neurodegenerative disorders were expected.

Although many questions in conversion of rPrP to PrP^{res} are to be answered, the Fe(III)-associated alterations in gene expression are important step in further understanding of the molecular mechanisms underlying prion-related disease pathogenesis. Other extended studies that include similar expression patterns in animal models are needed to confirm functions of each gene *in vivo*.

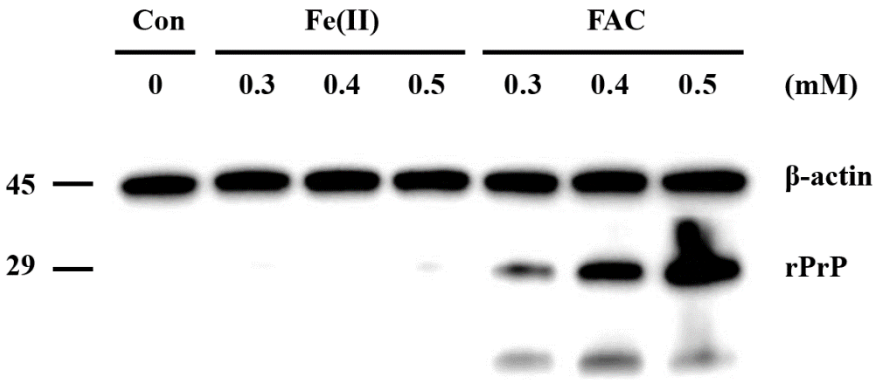
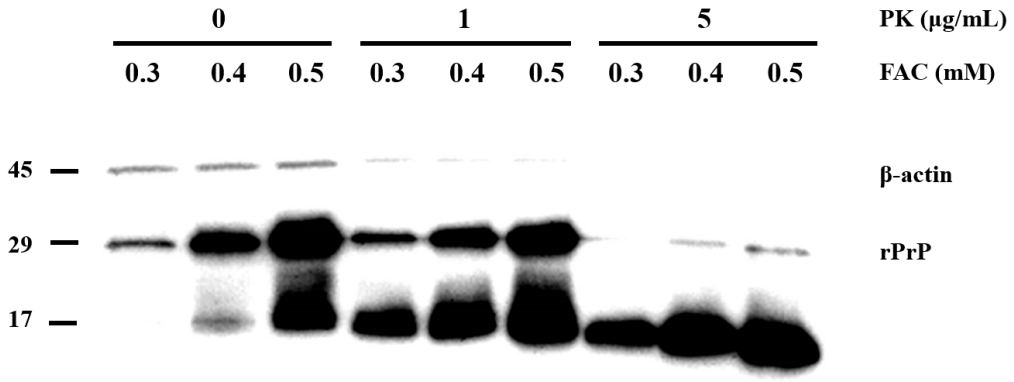


Figure 1. Accumulation of internalized rPrP with FAC.
Cells were treated with 0.3–0.5 mM of either $FeCl_2$ or FAC for 24 h. Subsequently, 0.6 μM rPrP was added for another 24 h. The internalized rPrP was detected with the 1E4 antibody. Internalized rPrP accumulated in an iron concentration-dependent manner.



Figiure 2. Conversion from rPrP to PrP^{res} with FAC.
PK concentration-dependent digestion of internalized rPrP induced by different concentrations of FAC.

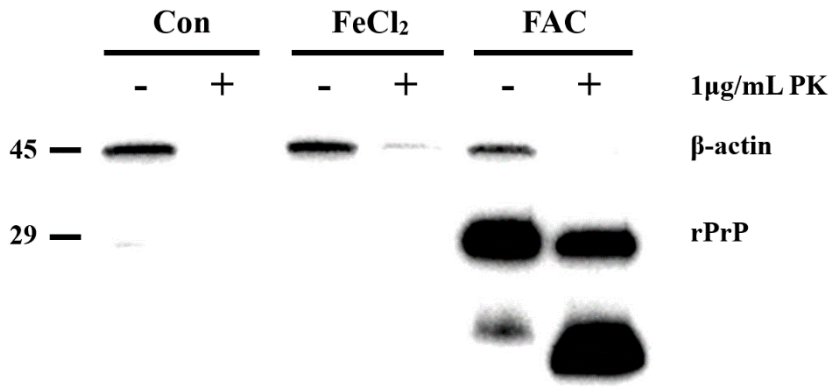


Figure 3. Accumulation of PK-resistant internalized rPrP.
The levels of internalized rPrP accumulated under either $FeCl_2$- or FAC-treated conditions were compared according to PK treatment (+: with PK treatment; -: without PK treatment).

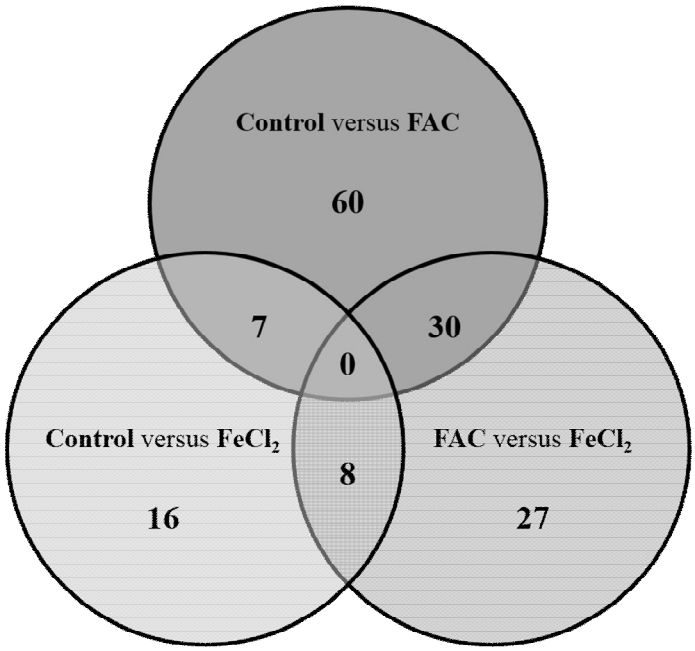


Figure 4. Venn diagrams illustrating differentially expressed genes.
Differentially regulated genes (≥ 1.5-fold change in expression) in three comparisons (control versus FAC, control versus FAC, and FAC versus $FeCl_2$) were merged. The number of differentially regulated genes in FAC-exposed samples was significantly higher than the number of upregulated genes from $FeCl_2$-treated samples. Only 30 genes in the overlapping circle of control versus FAC and FAC versus $FeCl_2$ were filtered as significantly altered genes in the sample treated with FAC.

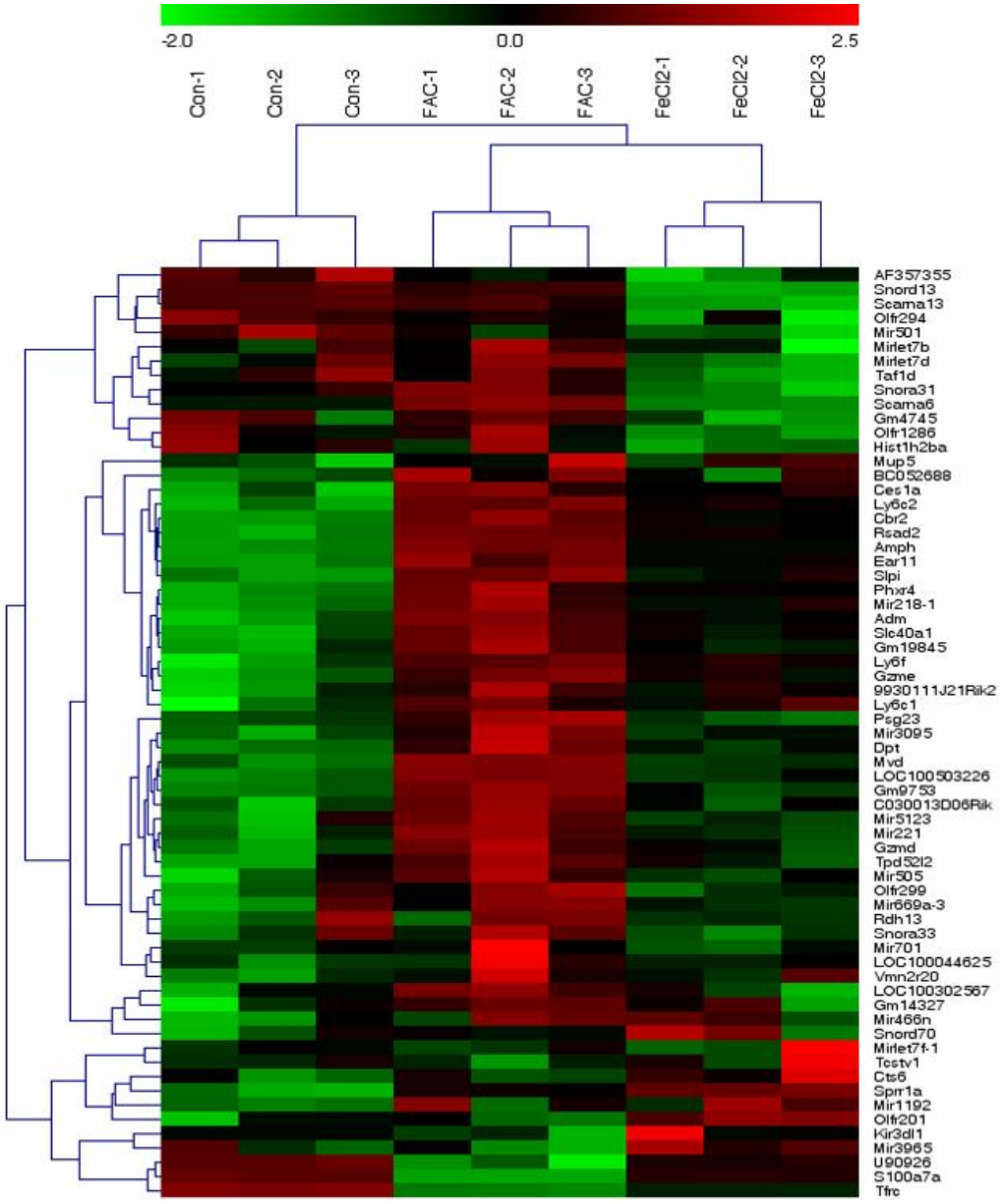


Figure 5. Hierarchical cluster of differentially expressed genes.
The horizontal axis represents a gene, and the vertical axis represents the information for each sample. The color represents the level of expression. Consistent patterns of differentially expressed genes were observed when the negative control and FAC-treated samples were compared.

Table 1. Upregulated genes in FAC-exposed cells

Gene Symbol	Gene Name	Fold Change	adj. *P*
	Vesicle-mediated transport / Synapse		
Amph	amphiphysin	1.540554775	0.000894778
Slc40a1	solute carrier family 40 (iron-regulated transporter), member 1	1.601993471	0.015480088
	Cell death		
Gzme	granzyme E	2.251201127	0.015480088
Gzmd	granzyme D	1.968008238	0.025681381
Slpi	secretory leukocyte peptidase inhibitor	1.567382505	0.001637086
	Cell proliferation		
Adm	adrenomedullin	1.545467258	0.015480088
Dpt	dermatopontin	1.500469054	0.025790173
	Immune response		
Amph	amphiphysin	1.540554775	0.000894778
Ly6f	lymphocyte antigen 6 complex, locus F	1.970531676	0.025681381
Ly6c1	lymphocyte antigen 6 complex, locus C1	1.81769205	0.053215295
Ly6c2	lymphocyte antigen 6 complex, locus C2	1.632726294	0.003272265
Rsad2	radical S-adenosyl methionine domain containing 2	1.547814589	0.001418653
Slpi	secretory leukocyte peptidase inhibitor	1.567382505	0.001637086
	Cell signaling		
Mvd	mevalonate (diphospho) decarboxylase	1.572491328	0.001637086
Cbr2	carbonyl reductase 2	1.504005207	0.001770869
9930111J21Rik2	RIKEN cDNA 9930111J21 gene 2	1.901950921	0.051220397
	Extracellular activity		
Slpi	secretory leukocyte peptidase inhibitor	1.567382505	0.001637086
Mup5	major urinary protein 5	1.559487225	0.162065499
Adm	adrenomedullin	1.545467258	0.015480088
Dpt	dermatopontin	1.500469054	0.025790173
	Metal ion binding		
Slc40a1	solute carrier family 40 (iron-regulated transporter), member 1	1.601993471	0.015480088
Rsad2	radical S-adenosyl methionine domain containing 2	1.547814589	0.001418653

Significantly upregulated genes are listed. This gene group comprises vesicle-mediated transport genes, synapse genes, cell death genes, cell proliferation genes, immune response genes, cell signaling genes, extracellular activity genes, and metal ion-binding genes.

Table 2. Downregulated genes in FAC-exposed cells

Gene Symbol	Gene Name	Fold Change	adj. *P*
	Metal ion transport		
S100a7a	S100 calcium binding protein A7A	1.694439055	0.000028
Tfrc	transferrin receptor	2.054890896	0.000028

Significantly downregulated genes are listed. This gene group comprises metal ion transfer genes.

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국문초록

Fe(III)에 의한 프리온 단백질 변성 관련 유전자 발현 양상

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프리온 단백질의 변성은 전염성 해면양뇌증 (Transmissible spongiform encephalophathies; TSEs)을 포함한 만성퇴행성 뇌질환의 주된 현상이다. 프리온 관련 질병에서 철 이온의 증가가 관찰되는데 이는 프리온 단백질이 철 저장체인 ferritin 과 응집을 이루기 때문으로 여겨진다. 특히 프리온 단백질의 응집과 변성은 이가 철이 아닌 삼가 철 특이적으로 일어난다. 삼가 철에 의한 프리온 단백질의 Proteinase K (PK) 분해 저항성 습득은 프리온 단백질과 삼가 철 사이의 단순 반응이 아닌 좀더 복잡한 세포내 환경을 요한다고 제기된 바 있다. 이 논문에서는 삼가 철에 의해 발생한 프리온 단백질 변성을 마이크로어레이를 이용하여 유전자 발현 수준에서 분석하였다. 삼가 철에 노출된 세포에서 총 97 개의 유전자가 다르게 발현되었고, 그 중 85 개의 유전자는

upregulation 을, 12 개의 유전자는 downregulation 을 나타냈다 (≥1.5-fold change). 그러나 이가 철 노출 환경에서의 세포는 눈에 띄는 유전자 발현 양상을 찾을 수 없었다. 식별된 유전자들을 각 기능별로 분류해본 결과, 세포성장과 유지 또는 세포 내·외적 운반 환경에 높은 관련이 있는 것으로 관찰되었다. 이러한 결과는 삼가 철이 레덕스 메커니즘에 의한 프리온 단백질 변성 관련 유전자 발현에 영향을 미칠 것이라고 유추할 수 있었다. 이같은 신경세포 내 유전자들의 변화된 발현 분석은 프리온 질병 또는 다른 관련 질병의 발생과 진행에서 나타나는 프리온 단백질 변성 메커니즘에 대한 이해를 제공 할 것이다.

주요어: 철 이온, 변성, 프리온질병, 마이크로어레이, 유전자 발현, 레덕스

학 번 : 2012-21536