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Molecular Basis of Allogeneic Pluripotent Stem Cell Treatment of a Patient with Sporadic ALS

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Abstract

There are no curative strategies for ALS, and as a result the alternative therapies are gaining ground, in particular the stem cell-based therapies which hold a promising future. Here we describe an ALS patient, who by choice, received allogeneic human cord-blood derived multipotent stem cells (MSC) intravenously and intrathecally. This is the first snapshot of pre-and post-stem cell transplantation in an ALS patient through transcriptomic, epigenomic, and proteomic follow-up elucidating the effect of stem cells in ALS. Although new CSF proteins (FGA, B2M, FN1 and IGFBP3) were observed, but more work is needed to fully

elucidate the effect based on larger cohorts in a clinical trial setting. These analyses may serve as a future guide to both scientists and clinicians alike in defining the utility of such cells in ALS treatment.

Keywords
Stem cells; pluripotent stem cells; transcriptome; umbilical cord-derived stem cells; multipotent stem cells (MSC); proteome; micro-RNA (miRNA)

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal neurodegenerative disease that causes the death of motor neurons leading to muscle wastage and eventual paralysis. Death usually occurs 3 to 5 years post diagnosis [1]. The sporadic form (sALS) occurs in approximately 90% of all ALS cases and cannot be explained by familial inheritance of a specific mutation [2]. There are currently very few treatment options available for ALS, with most providing only transitory symptomatic relief. This is mainly attributed to, and complicated by the heterogeneity of sALS in terms of both clinical and molecular presentation [3-5]. Riluzole is currently the only drug for the treatment of ALS, which extends the survival of ALS patients by an average of 3 months, but its mechanism of action remains unclear with variable patient outcomes [6]. Thus, there is a clear lack of therapies that can provide long-term relief, and augment the life span of ALS patients.

Personalized medicine approaches similar to those favored by many cancer researchers [7] hold promising future in the field of personalized medicine. But before these personalized genomic approaches become available, stem cells are being considered as an alternative treatment given that they are well tolerated when delivered intravenously or intrathecally. Researchers have been using many types of stem cells, including induced pluripotent stem cells (iPSCs), which may be among the most promising of cells with a potential for treatment. Both iPSCs and Multipotent stem cells (MSC) which hold the potential to develop into neural stem cells remain under investigation for neurodegenerative diseases [8].

To date, three clinical studies with NurOwn® (the MSC-NTF-mesenchymal stem cell-neurotropic factor cell-therapy) in ALS patients have been completed. The first two open-label studies, confirmed that the treatment was safe and well tolerated when administered intrathecally (IT) or intramuscularly (IM), as well as by combined IT and IM administration [9]. These studies demonstrated preliminary efficacy, and by retarding the rate of disease progression. Another Phase 3 clinical trial (NCT03280056) for NurOwn is underway.

Although the NurOwn trial is the first stem cell-based clinical trial in ALS, and also the first to claim the efficacy of stem cells in ALS treatment, there is no evidence from other sporadic trials replicating similar effects in slowing the rate of disease progression of ALS (reviewed in [8]). Despite this, a number of clinics in different parts of the globe continue to provide SCs as an alternative treatment to vulnerable ALS patients without any follow-up defining clinical outcomes. As a result, the effects of SC treatments remain obscure.

In this study, we have comparatively visualized expression changes that occur pre-and post-SC administration of pluripotent allogeneic stem cells, and carried out a deeper analysis of

transcriptome (mRNA), miRNA (small 20 nucleotide non-coding RNA) and proteome in peripheral blood mononuclear cells (PBMCs), and cerebrospinal fluid (CSF), from an ALS patient who opted for huCB-MSCs treatment. This is the first snapshot of functionally meaningful integrated transcriptomic and proteomic changes in an ALS patient upon allogeneic SC treatment. We believe these molecular changes reflect patient-specific changes that are related to allogenic stem cells, and possibly to ALS, thereby ensuring a solid foundation for learning lessons for future clinical translation through better optimization and innovation of stem cells before they are considered for the treatment of ALS, and other neurodegenerative diseases (NDs).

2. Materials and Methods

2.1 Patient Samples and Clinical Profile

The ALS patient described herein presented with lower limb-onset ALS. The samples collected in this study were comprised of both blood and CSF (Cerebrospinal fluid) samples donated upon written consent by the patient collected prior to-and three days after the pluripotent stem cell treatment. The pluripotent stem cells, derived from human cord blood, were administered both intrathecally and intravenously, with 5 injections each over 9 days. The CSF samples were stored at -80°C until they were required for use in various molecular profiling experiments. The blood samples were immediately processed using FICOLL gradient to isolate PBMCs (peripheral blood mononuclear cells), which were then stored at -80°C until required for use in molecular profiling experiments.

2.2 Phenotypic Determination of Pluripotent Stem Cells Derived from Human Cord Blood

Umbilical cord tissue was procured from the hospital. Informed consent was acquired prior to delivery. Maternal blood was tested for HIV, hepatitis B, hepatitis C, syphilis, HTLV (human T cell leukemia virus), CMV (cytomegalovirus) and bacterial contamination. Umbilical cord tissue was sterilised prior to isolation with povidone-iodine solution in a biosafety cabinet in a clean room. Cells were isolated on plate. Media was changed every 3 days and cells were monitored for cell growth and morphology. Once the cells had reached 70% confluence on the plate, they were passaged and reseeded to more plates. When the cells were adequate for transplantation, they were counted for viability and cytologic markers Characterization of hUCB-MSCs was carried out by immunophenotyping using both MSC-positive and MSC-negative surface markers. Briefly, 60 to 80% confluent flasks of expanded MSCs were trypsinised, followed by washing with 1× PBS and fixed in 4% paraformaldehyde for 15 minutes at 4°C. Cells were then incubated with FITC/PE-conjugated CD73 (1:100; BD Pharmingen, San diego, CA, USA), CD44 (1:100; BD Pharmingen), CD45 (1:100; BD Pharmingen), CD105 (1:100; BD Pharmingen) and CD29 (1:100; BD Pharmingen) primary antibodies in the dark at 4°C for 1 hour and finally resuspended in 1× PBS containing 3% BSA for fluorescence-activated cell sorting analysis. To avoid non-specificity and background staining, appropriate isotype secondary antibody controls (mouse IgG-Att488 and mouse IgG-PE) and cell-only controls were used. A total of 10,000 events were analyzed using a BD FACS Aria Flow Cytometer (BD Biosciences, San Jose, CA, USA). Culturing media was sent for sterility, mycoplasma and endotoxin tests prior harvesting cells for transplantation. Cells were washed with 37°C normal saline solution to remove as much media as much as possible. Prepared cells were

placed in glass vials and transported to clinical site. The ethics for the stem cell part was fully covered by the institutional ethics at the Unique access Medical, Bangkok, Thailand where this stem cell treatment was performed.

2.3 Stem Cell Therapy

The patient was hospitalized for 14 days at the UAM, Bangkok, Thailand, which acted as a facilitator for the stem cell treatment the study patient received through a third party. The treatment comprised of IV infusions of vitamins, growth factors and anti-histamines. The stem cells were injected intravenously (IV) and intrathecally every 3 days, over 9 days. The patient received 5 applications of hUCB-MSCs at days 2 (30 million IV), 4 (30 million intrathecal), 8 (35 million intrathecal), 11 (35 million intrathecal) and 13 (30 million IV). A total of 160 million stem cells were transplanted over a course of 5 injections.

Intravenous administration of hUCB-MSCs was performed as follows; 2 injections containing 30 million stem cells each. 5mg Chlorphenaramine maleate was administered by IV route 30 minutes before each injection. Stem cells were mixed with 50cc 0.9% normal saline and transfused at a rate of 4cc/minute using infusion pump (Terumo Terufusion Syringe Pump TE-331). The intrathecal injection protocol was performed over 3 injections containing 30 million, 35 million, and 35 million stem cells, respectively. 5mg Dexamethasone was administered by IV route 1 hour before each injection. A lumbar puncture was performed between L3 and L4 vertebral levels using spinal needle (LASA 27G) and a total of 3cc volume was injected into the CSF.

The blood samples were processed for the collection of cells and plasma. All samples of CSF, blood cells and plasma were stored at -80°C until processed for transcriptomic (mRNA and miRNA) and proteomic analysis.

The patient is an ALS sufferer and is looking for possibly viable and meaningful new treatments. This is why the patient chose to do the stem cell infusion treatment at the Unique Access Medical, Bangkok, Thailand. Since the patient wanted to know through a follow-up, which UAM does not provide, he asked the IGO (Iggygetout) to perform the follow-up genomic analysis to delineate the effect of stem cells pre-and post-treatment. Thus, it was patient's choice to opt for the treatment and get his samples analyzed for which full written consent was obtained from the patient prior to this work. All medical procedures were done under the guidance of highly qualified doctors at the UAM, Bangkok, Thailand.

2.4 RNA Expression Profiling

All profiling studies were performed pre-and post-SC treatment from the study patient. Several different techniques of RNA expression profiling were used throughout this study. For the CSF, MIRXES (now ARK Biosciences, Singapore) absolute quantification qPCR was used to analyze the levels of 102 DE miRNAs and was complemented by complete transcriptomic miRNA sequencing using RNAseq, performed at LC Biosciences, Boston, USA. The same procedure was attempted for plasma but several samples failed quality control (QC), so this procedure was abandoned in favor of small RNA sequencing from PBMCs, which was performed at the Beijing Genomics Institute (BGI), Shenzhen, China. The same organization also performed mRNA transcriptome sequencing from the PBMCs using RNAseq on the BGI-500 platform. With these data sets, we performed differential expression analysis to identify DE miRNAs in patient's CSF and PBMCs pre-and post-

huCB-MSC treatment. In contrast, the mRNA expression profiling was only performed in PBMCs, as mRNA transcriptome is challenging from the CSF.

For mRNA sequencing data, adaptors were trimmed by Trimmomatic v0.38 [10], sequences were aligned to GRCh38 with STAR-2.6.1 [11] and gene count was qualified by featureCounts v1.6.3 [12].

Raw miRNA reads were subjected to LC Biosciences's in-house program, ACGT101-miR, to remove adapter dimers, junk, low complexity, common RNA families (rRNA, tRNA, snRNA, snoRNA) and repeats. Subsequently, unique sequences with length in 18~26 nucleotide were mapped to specific species precursors in miRBase 22.0 by BLAST search to identify known miRNAs and novel 3p- and 5p- derived miRNAs.

For mRNA and miRNA data, since no duplicate sample is available, current mainstream software [13-15] was not used for differential expression analysis, as they all depend on multiple samples for the estimation of expression variation. We thus identified differentially expressed genes by firstly selecting distributions that best fit the expression changes, then selecting genes that deviated from the distributions as differentially expressed (Figure S1). To find the distributions that best fit patient's data, we denoted the expression level of gene g at pre-treatment and post-treatment to be $X_{g\text{-pre}}$ and $X_{g\text{-post}}$, respectively, and the $\log_2$ fold change of gene g was defined as $\log_2(FC_g) = \log_2(X_{g\text{-post}}/X_{g\text{-pre}})$ then the distribution of $\log_2(FC_g)$, for all $g \in G$, where G denoted all mRNA or miRNA genes, was explored by Cullen and Frey graph (Figure S2) [16], to visualize the similarity distribution of several candidate genes in the context of patient's data (Figure S1). Logistic and normal distribution showed the most similarity with patient's distribution. We selected normal distribution to describe our data, as we supposed that the longer tails deviated from the distribution were enriched with genes affected by the treatment.

Since the genes not affected by the treatment should change symmetrically, a mean value of zero was used as the mean of distribution. To better estimate the standard deviation (SD) of the distribution, considering that genes with similar level of expression have higher possibility of having similar standard deviation [13, 14], SDs were estimated from other genes with similar level of expression. We also noticed that SD could be gene-specific, which can be estimated from SD of the same gene in control samples. So, for gene g, its SD was estimated as

$$SD_g = SD_{g\text{-win}} + SD_{g\text{-control}}$$

Where $SD_{g\text{-win}}$ is the standard deviation of $\log_2$ fold-change of 500 genes (100 genes for miRNA) with closest expression level to gene g, accounting for the variation of SD in different levels of expression (Figure S3). Values outside of 0.02 and 0.98 quantiles were enriched with extreme data and could be potential differential expressions, so they were viewed as outliers and excluded in $SD_{g\text{-win}}$ estimation. And then, $SD_{g\text{-control}}$ is the SD of the same gene in control samples, representing the impact of gene specific variation. $SD_{g\text{-control}}$ was scaled to center at zero and have the same standard deviation as $SD_{g\text{-win}}$. The p value of each $\log_2(FC_g)$ under the distribution was calculated, and multiple testing was adjusted by Benjamini–Hochberg method [17], genes with adjusted p value < 0.05 and absolute $\log_2$ fold change > 1.0 were considered to be differentially expressed.

Pathway Overrepresentation Enrichment Analysis on the differentially expressed mRNA genes

and proteins was performed with WebGestaltR package [18], using KEGG database [19].

2.5 Integration of miRNA and mRNA Data from PBMCs

An mRNA-centric approach was used to integrate mRNAs, miRNAs and proteins, to identify most confident set of dysregulated mRNAs whose differential expression was supported by the expression of their regulating miRNAs or protein products. Thus, for a differentially expressed mRNA j, a confidence score was derived as below

$$Score_{mRNA\text{-}j} = \beta1*\log2(FC_{mRNA\text{-}j}) + \beta2*\sum(-\log2(FC_{miRNA\text{-}*j})) + \beta3*\sum\log2(FC_{protein\text{-}*j})$$

Where $FC_{mRNA\text{-}j}$ is the fold-change of mRNA j, and $\sum(-\log2(FC_{miRNA\text{-}*j}))$ is the sum of -log2 fold-change of all differentially expressed miRNAs targeting the mRNA, and this term was normalized to make all mRNAs have a mean absolute value of 1.0 on this term. Similarly, $\sum\log2(FC_{protein\text{-}*j})$ is the sum of log2 fold-change of all the DE mRNA's and translated proteins and was also normalized to make all sums have a same mean value. $\beta1$, $\beta2$ and $\beta3$ are coefficients, which can be assigned to different values to change the contribution of each term to the score, here we used $\beta1=1$, $\beta2=3$ and $\beta3=3$, considering the first term $\log2(FC_{mRNA\text{-}j})$ had a mean absolute value of around 3.0 in our patient.

2.6 Proteome Profiling

In this study, proteome profiling also was performed only from the CSF compartment because of its relevance in ALS and other NDs. Proteomic experiments were performed using a 5600 TripleTOF mass spectrometer (Sciex, Framingham, MA) coupled to an Eksigent Ultra-nanoLC-1D system (Eksigent Technologies, Dublin, CA). SWATH peaks were extracted using PeakView (v.2.2). Shared and modified peptides were excluded. Peak extraction parameters were set as the following: 100 peptides per protein, 6 transition ions per peptide, peptide confidence threshold 99%, FDR extraction threshold 1%, XIC (Extract Ion Chromatogram) retention time window 5 min and mass tolerance 75 ppm. Proteome analysis was performed on CSF samples only. The R package DEP [20] was used to identify differentially expressed proteins. Proteins with adjusted P value < 0.05 and absolute log2 fold change > 0.5 were considered to be differentially expressed.

3. Results

3.1 MicroRNA and mRNA Expression in PBMCs Post-Stem Cell Treatment

As the stem cell administration was performed both intravenously and intrathecally for the study patient, several analyses were performed to determine the nature of transcriptional changes occurring pre-and post-SC treatment in both PBMC and CSF compartments.

Firstly, the mRNA expression profiling was conducted on the PBMCs, and the DE gene expression profiles appearing post-SC treatment are shown in Figure 1, where we used a model with gene wise expression variation estimated from six healthy individuals (*see Materials and Methods*). Subsequently, a similarly structured analysis of miRNA expression from these PBMCs was also performed. Both datasets were first analyzed individually, and then an integration of the mRNA and miRNA datasets was performed to reveal gene targets of each of the DE miRNAs.

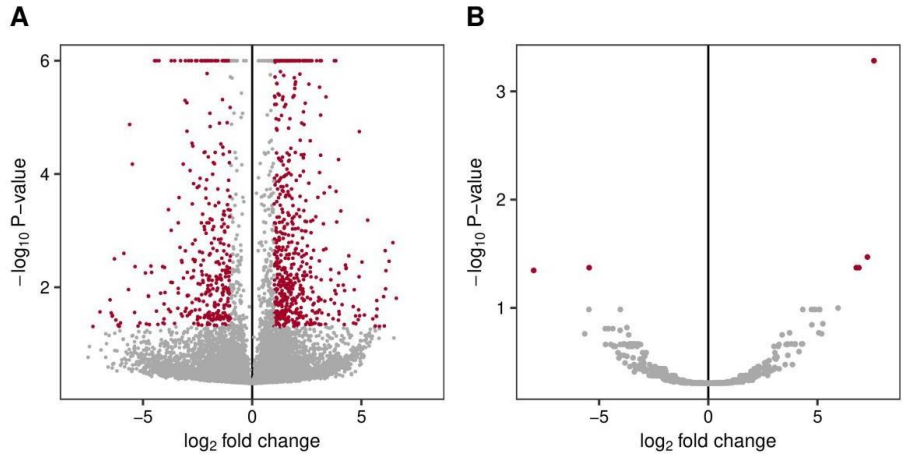

Figure 1 Volcano plot showing differentially expressed mRNA (A) and miRNA (B) genes in PBMC after SC treatment. Data for genes that were not classified as differentially expressed are plotted in grey. In red, we plotted data for genes that are differentially expressed after treatment (Benjamini–Hochberg corrected P value < 0.05 and absolute log2 fold change > 1.0).

Although the up-regulated mRNAs post-treatment had a broad array of functions, most statistically significant pathways according to the GSEA were largely focused in the area of the oxidative phosphorylation pathway. The inclusion of the neurodegenerative disease pathways (Alzheimer's disease, Huntington's disease, Parkinson's disease) is due almost entirely to the presence of these genes, with a handful of exceptions in the Alzheimer's disease KEGG pathway. Lysosomal pathways also seem to be strongly activated post-SC treatment due to the up-regulation of several cathepsins (CTSG, CTSS, CTSH, CTSZ) amongst other genes. There was also an up-regulation of many major histocompatibility complex 2 (MHC2) components which were associated with Leishmaniasis, asthma, intestinal immune network for IgA, Allograft rejection and graft versus host disease (Table 1). This concurs with the immunological response to a foreign body, as the SCs study subject received were allogeneic.

Table 1 GSEA of genes up-regulated post-SC treatment.

Description	# Genes in Gene Set (K)	# Genes in Overlap (k)	k/K	p-value	FDR q-value
Alzheimer's disease	169	22	0.1302	9.73E-17	**1.81E-14**
Huntington's disease	185	20	0.1081	8.94E-14	**8.32E-12**
Parkinson's disease	133	17	0.1278	4.12E-13	**2.56E-11**
Oxidative phosphorylation	135	16	0.1185	6.58E-12	**3.06E-10**
Lysosome	121	13	0.1074	2.13E-09	**7.94E-08**
Leishmania infection	72	10	0.1389	1.27E-08	**3.93E-07**

Asthma	30	7	0.2333	4.73E-08	**1.26E-06**
Intestinal immune network for IgA production	48	8	0.1667	8.50E-08	**1.98E-06**
Allograft rejection	38	7	0.1842	2.71E-07	**5.59E-06**
Graft-versus-host disease	**42**	**7**	**0.1667**	**5.55E-07**	**1.03E-05**

The Reactome analysis further confirmed the preponderance of immunological pathways, given that these were the only pathways that were statistically significant according to FDR (FDR<0.05). Of the pathways highlighted by the Reactome analysis, it appears that neutrophil degranulation and T cell receptor (TCR) pathways were most likely induced post-stem cell administration (S4: Reactome report).

The GSEA of the down-regulated genes post-SC treatment was far less far less congruous with neurodegenerative disease and immunological pathways (Table 2). Almost all of the highlighted pathways were represented in cancer, except for some pathways (adherens junction, regulation of actin cytoskeleton, and ErvB signalling, etc) that are commonly shared between cancer and NDs. Reactome analysis was more revealing, strongly indicating loss of DNA repair functions, especially of the D-loop resolution by multiple mechanisms, and this process may be under transcriptional control by TP53.

Table 2 GSEA of down-regulated genes post SC treatment.

Description	**# Genes in Gene Set (K)**	**# Genes in Overlap (k)**	**k/K**	**p-value**	**FDR q-value**
Pathways in cancer	328	16	0.0488	5.45E-10	1.01E-07
Cell cycle	128	9	0.0703	1.60E-07	1.49E-05
Endocytosis	183	10	0.0546	3.47E-07	2.15E-05
ErbB signaling pathway	87	7	0.0805	1.58E-06	6.84E-05
Prostate cancer	89	7	0.0787	1.84E-06	6.84E-05
Pancreatic cancer	70	6	0.0857	6.18E-06	1.91E-04
Adherens junction	75	6	0.08	9.23E-06	2.45E-04
Regulation of actin cytoskeleton	216	9	0.0417	1.23E-05	2.85E-04
Endometrial cancer	52	5	0.0962	2.14E-05	4.42E-04

Riboflavin metabolism	16	3	0.1875	1.38E-04	2.56E-03

3.2 Study Patient Derived mRNA and miRNA Data Integration

To gain functional insights pre-and post-stem cell treatment, the study patient-derived mRNA and miRNA datasets were integrated. Integration of miRNA and mRNA datasets from PBMCs revealed an interesting trend that appeared to be driven by the loss of hsa-miR-7-1-3p, which experienced an 8- fold decrease following SC treatment, allowing for greater expression of genes targeted by hsa-miR-7- 1-3p. Of the genes that are potentially targeted by miRNAs according to the data integration analysis, approximately 68% were regulated by hsa-miR-7-1-3p alone. It appears that loss of this miRNA produces an up-regulation of genes involved in calcium homeostasis, given the increase in FCGR2A and FCER1G alongside the hsa-miR-7-1-3p facilitated increase of Calmodulin 2. The target genes of hsa-miR-7-1-3p are shown in Table 3.

Table 3 De-repression of specific genes due to hsa-miR-7-1-3p decrease.

mRNA	Confidence Score	Gene log2FC
UBE2Z	7.402299	4.059224
TMEM64	6.700823	3.357748
USP14	8.699393	3.075713
TAB2	5.841999	2.498924
KCTD12	5.817337	2.474262
MICB	5.535705	2.19263
SSSCA1	5.47689	2.133815
RAB1A	5.337867	1.994793
C1orf52	5.263137	1.920062
PPP3R1	5.193411	1.850336
MCL1	5.154436	1.811361
TBC1D1	4.996831	1.653756
ATP5G3	4.995216	1.652141
CALM2	4.940433	1.597358
SNW1	4.78661	1.443535
IRF2BP2	4.707604	1.364529
ACTR2	4.630389	1.287314
PAFAH1B2	4.578266	1.235191
SUMO3	4.575015	1.23194
APH1A	4.449459	1.106384
PSME3	4.246237	0.903162
MAPRE1	4.146983	0.803908
PHF20	4.104277	0.761202
EIF4G2	3.979559	0.636484
LITAF	3.777569	0.434494
PDXK	3.759181	0.416107

PIK3CD	3.710616	0.367541

3.3 CSF Proteome

CSF proteome analysis was performed to derive information on the nature of proteins post-SC administration intrathecally, and their functional significance. Interestingly, a handful of statistically significant DE proteins which were up-regulated post-SC treatment in the study patient (Table 4, Figure 2 and Figure 3). For the most part, these proteins were the components from the complement system, specifically of complement C1 (C1QA, C1R) and C8. The remaining genes appear to be largely involved in modulating cell adhesion through interactions with extracellular matrix (ECM1, TIMP1, TGFBI) or cell motility (ACTG1). FCGBP is currently poorly annotated, and its role in CSF is not known.

Table 4 Differentially expressed proteins in CSF post SC treatment.

Gene	log2FC	adjusted p value
TIMP1	3.41578064	7.30E-10
TGFBI	2.55623672	1.12E-14
FCGBP	2.22464224	1.70E-06
C8B	1.87684549	1.94E-06
ECM1	1.51111486	0.029589225
C1QA	1.38421399	0.046050162
C1R	1.27545136	2.11E-05
ACTG1	1.23437432	0.038242275
APOC1	1.17366852	0.004134199
C1QB	1.03600085	0.016827174
LUM	0.95257067	0.006953647
FN1	0.9475261	0.00311977
C1S	0.8151554	0.005179625
C1QC	0.73354534	0.037721641
C7	0.6653083	0.04712592
B2M	0.64634301	0.043724133
APOD	0.55310562	0.046423406
C4B	0.51665648	0.009917909
C4B_2	0.51665648	0.009917909
SERPIND1	-0.53568481	0.038187358
CLEC3B	-0.67441187	0.003881719
ITIH1	-0.8401468	0.00620583
FGA	-0.85472187	0.025904091
IGFBP3	-5.25986929	1.12E-14

NB: Log-fold change with – sign denotes downregulation of the gene and with no sign upregulation.

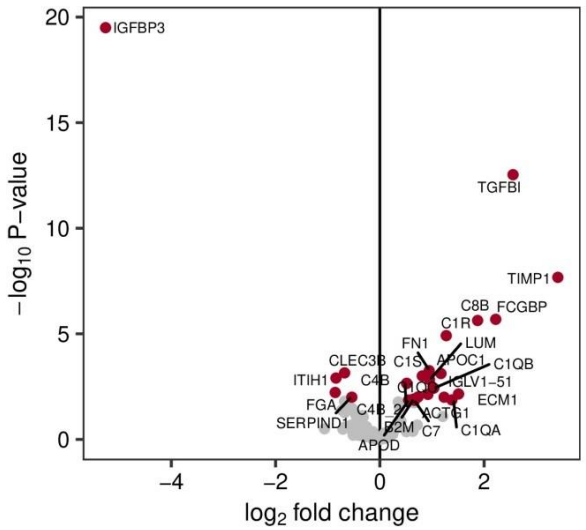

Figure 2 Volcano plot showing differentially expressed proteins in the CSF after SC treatment. Data for proteins that were not classified as differentially expressed are plotted in grey. In red, we plotted data for proteins that are differentially expressed after treatment (Benjamini-Hochberg corrected P value < 0.05 and absolute log2 fold change > 0.5). Symbols of gene encoding the proteins were labelled.

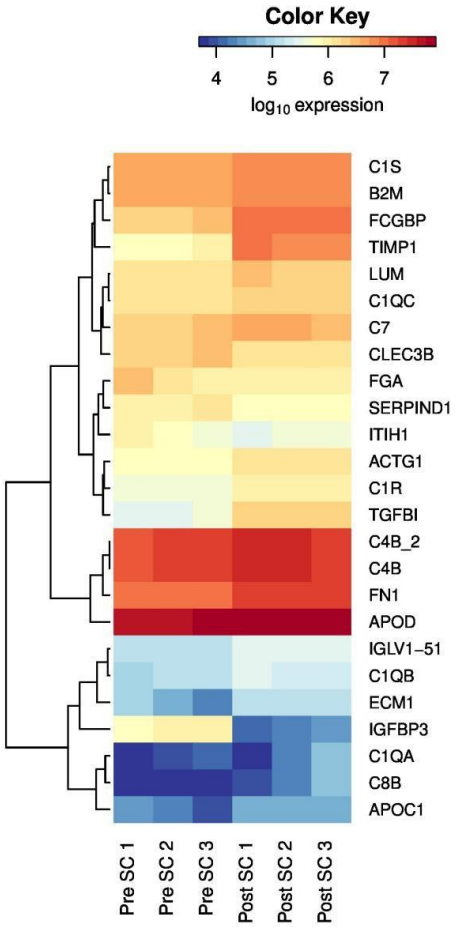

Figure 3 Heatmap of differentially expressed proteins in CSF after SC treatment. Three technical replicates were performed at both pre-treatment and post-treatment time points.

There were also a small number of down-regulated proteins in the patient's CSF post-SC treatment. The down-regulated proteins were also mostly made up of complement proteins again with several subunits of C1 (C1QC, C1QB, C1S), C7 and C4B. Fibrinogen alpha chain (FGA) and SERPIND1 were also down-regulated post-SC treatment and these genes also participate in complement and coagulation cascades. The remaining genes are largely secreted proteins involved in extracellular transport (CLEC3B, APOD), extracellular matrix processes (LUM, FN1 and hyaluronan binding (ITIH1). Of particular interest was the significant loss of IGFBP3 protein from CSF post-SC treatment. This protein may have the capacity to lower the bioavailability of IGF1, a neuroprotective protein. The loss if IGFBP3 may represent a shift in bioavailability of IGF1 post-SC treatment. At this stage it is difficult to comment on whether the loss of IGFBP3 is a positive or a negative change in terms of stem cell action.

4. Discussion

4.1 CSF Proteome Response to Allogeneic Stem Cell Infusion

It has become clear that many neurodegenerative diseases (NDs) share a number of genetic and epigenetic pathway disruptions. High throughput technologies allow the visualization of diverse genome entities (mRNA, miRNA, epigenome, proteome) and possible functional interactions between them occurring in different body fluids, including CSF, and in peripheral blood mononuclear cells (PBMCs) [21]. This facilitates multi-compartmental analysis which can be used in studying treatment response to therapy in a given compartment. In this case, allogeneic stem cell therapy response was recorded at the proteomic level for an ALS patient in the CSF. This is the first report to demonstrate the effect of allogeneic stem cell transplant and its genomic basis, which can shed light on its possible utility and define its relevance for future use in patients with neurodegenerative diseases.

Spinal cord is one of the primary sites of pathology in sporadic ALS [22-25], and because the patient received SC intrathecally, the CSF proteome was an intuitive choice to analyze the effect of allogeneic SC transplant. SWATH acquisition of the DE proteins showed that the most consistently dysregulated group of proteins were related to the complement and clotting cascades, with members fitting this description both up-regulated (C1QA, C8B and C1R) and down-regulated (C1QC, C1S, C7, C4B, FGA, SERPIND1). Unfortunately, the patterns of up-and down-regulation amongst this gene set did not indicate that the complement or coagulation pathways are overall hyper-or hypo-activated after SC treatment. However, fluctuations in the complement components C3 [26, 27] and C4d [28] have previously been demonstrated in the CSF of ALS patients, where both these components were up-regulated. Further, the inhibition of the complement system has also been proposed as a means of therapy in several neurodegenerative diseases including ALS [29]. C1QB and C1QC have also been identified as highly expressed in activated microglia in ALS and Alzheimer's disease patients, alongside B2M (a down- regulated protein post SC treatment) [30]. This phenomenon was also observed in the study patient's CSF where several complement components were down-regulated post-SC treatment, but a number of complement proteins were also up-regulated. Given the spread of up-and down-regulated complement components, it is difficult to determine whether a therapeutic, benign or exacerbating influence is being precipitated by SC treatment. Also, since this study is the first case study on genomic visualization of the effect of allogeneic SCs in an ALS patient, similar studies or

future clinical trials of allogeneic stem cell transplant will be required to further probe these observations and delineate their utility mechanistically in ALS.

Less ambiguous, however, is the down-regulation of FGA- a component of fibrinogen. Decrease in the amount of this protein may indicate less microvascular leakage into CSF from blood, as fibrin deposits are known to occur in ALS patients via this mechanism [31]. This will require further research to confirm whether this is a positive and appropriate metric for treatment response to allogeneic SCs in ALS patients and define its meaningful translation into treatments.

The decrease in beta-2 micro-globulin (B2M) protein may also indicate a positive therapeutic response as this is a well-defined pro-aging, anti-neurogenic factor that has been linked to decreased hippocampal health and function in mice and humans [32]. CSF B2M has also been used as a biomarker to differentiate between infection-related central neuroinflammation and patients without neuroinflammation, although this study was performed in pre-term babies [33]. The decrease in this factor post-SC treatment suggests a decrease in neuroinflammation and an increase in neurogenic properties, which may be useful in ALS as inflammation is the key modality that needs be reduced to overcome other challenges in the disease. Unfortunately, there is no literature relating directly to B2M modulation post-allogeneic stem cell treatment, and this is the first evidence in favor of allogeneic stem cells in an ALS patient. At this time, it is difficult to provide a meaningful metric for response to allogenic SC therapy in the study patient and comment on its actual utility in ALS patients. Nonetheless, these observations are in line with previous reports on the relevance of beta-2 micro-globulin in NDs, and provide possible future value in treating ALS with SCs but more research is needed in a clinical trial format.

There was also a decrease of apolipoprotein-D (Apo-D) protein following SC treatment, but its functional validity in terms of treatment response to SCs is difficult to comment on. The gene that encodes Apo-D is associated with aging neural tissue in both neurons and glial cells [34], and it is thought to be a neuroprotective antioxidant whose presence is beneficial during aging and some neurodegenerative diseases [35-37]. It has also been reported to be both up-and down-regulated in mice [38] and humans with ALS, respectively [39], but neither study was performed on CSF. There is currently no information regarding the behavior of APOD expression during a positive therapeutic response in any neurodegenerative disease. It is also difficult to interpret the increased level of Apo-C1 expression as either therapeutic or antagonistic. Most information regarding APOC1 in the brain comes from mutation studies as mutations are associated with various features of Alzheimer's diseases including age of onset or disease severity [40-42]. It is currently thought that Apo-C1 is linked with glial cell function during the Alzheimer's disease process [43] and it seems that APOC1 is enriched in aged microglia along with other risk factor genes for Alzheimer's disease [44]. However, nothing is known regarding the expression or role of Apo-C1 in sALS. While looking at the ratio of the Apo-D and APOC1 however, an interesting trend emerged. Elliot et al., have shown that Apo-D generally trends upwards as age advances, while Apo-C1 trends downwards as age advances [45]. Post-SC treatment, both of these trends seem to be reversed in this patient, which is of particular interest but the biological significance of this shift requires further study to be ascertained.

Another protein, IGFBP3 was the most down-regulated protein in the CSF after allogeneic SC treatment. In a previous study of ALS patient serum and CSF, it was shown that IGFBP3 levels did not differ between patients and controls [46]. IGFBP3 is a known competitive inhibitor for IGF1 [47], a neuroprotective protein [48] which has been suggested as a therapeutic option for ALS

[49]. Unfortunately, clinical trials of IGF1 could not demonstrate efficacy in ALS patients [50], and it has been suggested that interfering factors including IGFBPs are responsible for competitively inhibiting the added IGF1 protein, suppressing any therapeutic benefits it may have been able to exert [51]. However, IGFBP3 was not one of the IGFBPs that was associated with decreased free levels of IGF1, in ALS patients during these studies [51]. Nonetheless, evidence from this patient suggests that decreased IGFBP3 induced post-SC therapy may allow for enhanced activity of IGF1 in the CSF compartment. Any interaction between IGF1, IGF1R and IGFBP3 must be demonstrated in the context of ALS to support this finding as a positive outcome for therapy. Mechanistic data regarding how hUCB-MSCs induce this change would also be desirable in developing effective SC treatments for ALS.

The trends amongst the up-regulated CSF proteins were similar in that they described neither a fully neuroprotective or fully neurodegenerative response. TIMP1 was the most up-regulated CSF protein and a study by Lorenzl et al. has shown that TIMP1 is up-regulated in CSF from multiple neurodegenerative diseases, including ALS [52]. Further, the up-regulation of this gene in CSF is thought to be neuroprotective in multiple sclerosis patients [53], but it's down-regulation has also been reported in the CSF where it is a biomarker of Parkinson's disease, expressed at a ratio of 0.002 compared to healthy controls [54]. However, in plasma samples from sALS (sporadic ALS) patients, the TIMP1 expression levels did not differ significantly between patients and controls [55], thus it is likely that this gene is differentially regulated between plasma and CSF. Viewed in this light, TIMP1 up-regulation could be positively contributing post-SC treatment in the study subject. We hypothesize that this is possibly occurring in an astrocyte- dependent manner as it has been shown that TIMP1 modulates the response of astrocytes to IL1B [56] -a pro-inflammatory cytokine that is implicated in damaging neuroinflammatory processes in neurodegenerative diseases [57-59]. A greater understanding of the role of TIMP1 is necessary before we define the efficacy of treatment with allogeneic stem cells in ALS.

TGFBI was the next most up-regulated protein in the CSF and this is also a somewhat ambiguous finding. TGFBI is induced by TGFβ1, which has been found to be up-regulated in ALS patients before, and positively correlates with disease duration [60]. This is certainly the case with the study patient who has survived almost 8 years with the disease and the mean survival period is 3 – 5 years [61]. However, TGFBI is only indirect evidence of up-regulated TGFβ1, as TGFβ1 was not seen by the mass spectrometry analysis. Literature regarding the expression of TGFBI in ALS reveals a positive correlation with fast progressing ALS compared to slow progressing ALS [38], but this discovery was made in plasma samples and may not be particularly relevant to the findings of this case study. Also, our unpublished data on ELISA of TGFβ1 on patient's plasma also did not show any correlation. There are no other associations of TGFβ1 with any other neurodegenerative disease, either positive or negative, and its regulation post-SC treatment remains tantalizing.

FCGBP protein was also up-regulated in the CSF post-SC treatment, but its potential therapeutic benefit remains to be elucidated. It has been discovered in the motor cortex neurons [62] and spinal cord sections [63] from sALS patients in the past. Many of the remaining up-regulated CSF proteins are involved with extracellular matrix structure and function, and are also associated with ALS. For example, ACTG1 was found to be up-regulated in white blood cells from sALS patients [64], ECM1 was found up-regulated in the spinal cord of a SOD1 mouse model [65] and LUM was found to be down-regulated in spinal cord sections from sALS patients, possibly due to methylation of the gene in this tissue [66]. Fibronectin (FN1) has been found up-regulated in the

skin of ALS patients [67] but down-regulated in plasma [67]. Fibronectin is a neuroprotective protein that is capable of transactivating various growth factor receptors including IGF1R [68]. Coupled with the loss of IGFBP3, it is possible that SC therapy has yielded a decrease in inhibition and an enhanced response to IGF1. To determine if the modulation of these proteins with stem cells is truly therapeutic, further randomized clinical trials are needed to assess the therapeutic efficacy of allogenic mesenchymal stem cell treatment.

4.2 PBMC Response to Allogeneic Stem Cell Infusion

Given that SCs were also administered intravenously, the response of white blood cells was analyzed. This included a mRNA and miRNA focused approach, including an integration of the data sets to estimate which miRNA:mRNA target relationships are the strongest and most impacted by SC treatment. Hsa-miR-7-1-3p was the only miRNA that seemed to be causing significant dysregulation of a number of transcripts in this analysis. This miRNA has been found up-regulated in hippocampal regions of late onset Alzheimer's disease [69] and down-regulated in MS [69]. This miRNA was down-regulated post-SC treatment and appears to have resulted in de- repression of multiple mRNA transcripts related to NFAT activation and B and T cell receptor signaling according to Reactome and KEGG analysis. These processes are probably linked given the intrinsic role of NFAT in T cell activation [70, 71]. Furthermore, the broader gene expression patterns suggest general immunological activation, such as the increase in many components of the MHC2. This type of activity is associated with presenting antigens from foreign sources to induce immune activation [72]. The induction of lysosomal genes also supports the idea that immune cell activation is occurring post-SC treatment [73], as does the increase in mitochondrial components that occurs during immune activation of T cells [74]. Whether these effects are favorable for patient's health in the long term remains to be ascertained. We emphasize that even after two years post-SC treatment patient continues to be stable without any symptomatic decline, along with no deleterious or side-effects of the treatment.

An immune response, particularly a humoral one as is indicated by the presence of markers of activation in the PBMC population surveyed during this experiment. This is not unexpected given that humoral responses have been seen in multiple pre-clinical trials of multipotent stem cells (MSC) before [75]. However, the rapidity with which this response was observed (3 days post-SC treatment) does throw in to doubt whether these cells have had enough time to exert any kind of therapeutic impact. Given the lack of molecular measurements for therapeutic outcome however, this could not be confirmed.

5. Conclusions

A genomic snapshot of post-allogeneic stem cell treatment has never been derived from patients with ALS. This is the first report on the expression of genomic entities that come into play post-treatment and from which we can derive possible leads to their functionality, and possible therapeutic effect they incur. Although some of the differentially expressed proteins in the CSF suggest possible therapeutic response post-treatment, there is some ambiguity surrounding these molecular changes, which can be attributed to a complete lack of literature on the effect of allogeneic stem cell treatment follow-up at the genomic level. This ambiguity also reinforces the need for molecular markers of sALS that may be used to track disease progression. Moreover, it

also necessitates longitudinal analysis to determine how long these responses could be maintained and made durable

Although the allogeneic stem cells patient received was not a part of any ALS clinical trial, this case study illustrates the first follow-up at the genomic level of such a treatment. The power of performing integrated genomic analyses even on a single patient for deriving personalized data that can be of immense value in precision treatments and future interventions. This approach has certainly facilitated the stratification of differentially expressed miRNAs into those with no effect, those with some effect, and those with the greatest effect on the recorded phenotype. This is especially useful for sporadic and highly heterogeneous conditions like sALS, where it is difficult to identify the best possible disease-driving candidates. We believe this single case will inspire researchers to look into the allogeneic stem cell treatments more closely so that optimized, and possibly tailored made treatments can be offered in the future to the ALS sufferers. Whether such treatments will work optimally during early and late stages of ALS needs to be determined clinically.

Although stem cell-based therapies have generated widespread interest as a potential therapeutic approach, no conclusive results have yet been reported from clinical studies. While data from individuals/small cohorts appear to be encouraging, stem-cell-based therapies have not yet gained much traction because they don't yet represent a reproducible clinical option. There is a dire need of well-designed, randomized clinical trials with high reproducibility. Moreover, comparative studies that can address vital issues such as the nature, properties, number of autologous or non-autologous cells, the delivery route and rigorous clinical selection of patients who can clearly derive benefit from the cell-based therapies are sorely needed. Only this will provide the effective translation of research into the clinic [76].

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Additional Materials (if any)

The following additional materials are uploaded at the page of this paper.

1. Figure S1: Histogram distribution of $\log_2$ fold change of mRNA (A) and miRNA (B) expression in PBMC after SC treatment.
2. Figure S2: Cullen and Frey graph of $\log_2$ fold change of mRNA (A) and miRNA(B) expression in

PBMC after SC treatment, red circle was the observed mRNA (A) and miRNA(B) data.

3. Figure S3: Distribution of SD of $\log_2$ fold changes in sliding windows of 500 mRNAs (A) and 100 miRNAs (B) in the patient.

Author Contributions

NKS conceptualized, designed and executed the study from getting the SC trial done in Thailand, sample collection, processing, end-to-end genomic analysis, interpretation of results to writing the manuscript; SB assisted in writing some sections on reactome analysis, performing reactome analysis and miRNA analysis, MK directed the study; ZS and BL performed the genomic and miRNA bioinformatic analysis; XMS and MM supported the proteomic analysis and TZ and DP performed the proteomic bioinformatic analysis and helped with writing the analystical and methodology sections and provide data interpretation.

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Competing Interests

The authors have declared that no competing interests exist.

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