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Zika Virus Infection of Human Mesenchymal Stem Cells Promotes Differential Expression of Proteins Linked to Several Neurological Diseases

Walter O. Beys-da-Silva[1,2] · Rafael L. Rosa[2,3] · Lucélia Santi[1,2] · Markus Berger[2] · Sung Kyu Park[4] · Alexandre R. Campos[5] · Paula Terraciano[2] · Ana Paula M. Varela[6] · Thais F. Teixeira[6] · Paulo M. Roehe[7] · André Quincozes-Santos[8] · John R. Yates III[4] · Diogo O. Souza[8] · Elizabeth O. Cirne-Lima[2,9,10] · Jorge A. Guimarães[2,3]

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Abstract

The recent microcephaly outbreak in Brazil has been associated with Zika virus (ZIKV) infection. The current understanding of damage caused by ZIKV infection is still unclear, since it has been implicated in other neurodegenerative and developmental complications. Here, the differential proteome analysis of human mesenchymal stem cells (hMSC) infected with a Brazilian strain of ZIKV was identified by shotgun proteomics (MudPIT). Our results indicate that ZIKV induces a potential reprogramming of the metabolic machinery in nucleotide metabolism, changes in the energy production via glycolysis and other metabolic pathways, and potentially inhibits autophagy, neurogenesis, and immune response by downregulation of signaling pathways. In addition, proteins previously described in several brain pathologies, such as Alzheimer's disease, autism spectrum disorder, amyotrophic lateral sclerosis, and Parkinson's disease, were found with altered expression due to ZIKV infection in hMSC. This potential link between ZIKV and several neuropathologies beyond microcephaly is being described here for the first time and can be used to guide specific follow-up studies concerning these specific diseases and ZIKV infection.

Keywords Zika virus · Brain diseases · Human mesenchymal stem cells · Proteome · Microcephaly

Walter O. Beys-da-Silva, Rafael L. Rosa, and Lucélia Santi contribute equally for the manuscript.

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✉ Walter O. Beys-da-Silva
walter.beys@ufrgs.br

✉ Jorge A. Guimarães
guimar@cbiot.ufrgs.br

[1] Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2] Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil

[3] Programa de Pós-Graduação em Biologia Celular e Molecular, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[4] Department of Chemical Physiology, The Scripps Research Institute, La Jolla, CA, USA

[5] Proteomics Core, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA

[6] Centro de Cardiologia Experimental, Instituto de Cardiologia/ Fundação Universitária de Cardiologia, Porto Alegre, RS, Brazil

[7] Departamento de Microbiologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[8] Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[9] Programa de Pós-Graduação em Ginecologia e Obstetrícia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[10] Departamento de Patologia Clínica Veterinária, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

Introduction

Zika virus (ZIKV) belongs to the family Flaviviridae, genus *Flavivirus*, and is a single-stranded RNA virus that is currently getting major attention from the scientific community and health agencies around the world. The pathological potential of ZIKV was revealed in 2015 due to a microcephaly outbreak occurring in Brazil that was linked to its infection [1, 2]. Since then, ZIKV has been implicated into other neurological diseases worldwide.

Shotgun proteomics using Multidimensional Protein Identification Technology (MudPIT) [3] is a reliable way to characterize biological changes focusing on effects of environmental or physiological induction of gene expression. Differential proteomics has been applied to characterize molecular alterations promoted by various stimuli, such as drugs or pathological states. The rational use of proteomics is also widely applied to identify potential biomarkers of diseases and can reveal the molecular basis of host-pathogen interactions [3–6]. Unfortunately, despite the state of global health emergency on ZIKV disease declared by World Health Organization (WHO) and the intense attention of the scientific community since the Brazilian microcephaly outbreak, the potential of proteomics to understand ZIKV infections has been largely ignored. To our knowledge, only one paper has been published on global proteomics of ZIKV infection presenting interesting findings using an elegant and singular model of human neurospheres [7]. This lack of proteomics papers on ZIKV is surprising because this approach would be very helpful to identify genes, proteins, signaling pathways, and molecular processes linked to ZIKV's deleterious effects on different cells, animal models, or tissues.

In this report, human mesenchymal stem cells (hMSC) were infected with a Brazilian strain of ZIKV isolated during the Brazilian outbreak. hMSC are multipotent cells that have the capacity to differentiate into mature cell types including neurons [8], which are cells strongly affected by ZIKV infection [9]. Recently, it was shown that ZIKV has presented tropism to hMSC [10]. Microcephaly and the neurological disturbances observed in congenital ZIKV infections might be explained at least partially by an abnormal differentiation of infected stem/progenitor cells [10]. This postulation reinforces the urgent need of molecular information of stem cells infected by ZIKV. Our results present a substantial dataset containing hundreds of proteins from hMSC with expression altered due to ZIKV infection. Among these, we could molecularly assign several proteins with previously reported ZIKV clinical consequences like microcephaly and other well-known brain disorders, revealing dozens of new molecular targets for future studies.

Materials and Methods

ZIKV

The ZIKV strain 17 SM used in this work was isolated in 2016, in São Paulo city, Brazil, from a 33-year-old woman who reported fever with duration of 2 days, followed by headache and retro-orbital pain (days 2–3), and a maculopapular rash accompanied by an intense itching and diffuse arthralgia (on day 4). The 17 SM ZIKV strain was propagated in African green monkey kidney (VERO) cells (ATCC CRL-1586) and tittered expressed as plaque-forming units (PFU). Viral stocks were stored at − 80 °C until use.

Human Mesenchymal Stem Cell Cultures

After obtaining written informed consent, tissues were collected from three healthy donors at Hospital de Clínicas de Porto Alegre, Brazil, and cells were isolated [11]. The cell suspension was plated and maintained in culture in low glucose-Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) containing 1% penicilin-streptomycin (Gibco, Grand Island, NY, USA) and 20% fetal calf serum (Gibco, Grand Island, NY, USA) at 37 °C in a humidified 5% CO_2 atmosphere. The isolated cells developed to visible systematic colonies of adherent fibroblast-like cells at about 2–5 days after initial plating [11]. These cells were characterized by their adhesiveness, fibroblastoid shape, and ability to in vitro differentiate into adipocytes, osteocytes, and chondrocytes [12, 13]. Immunophenotypic identification for CD105, CD73, CD90, CD44, CD45, CD34, CD11b, CD19, and HLA-DR was performed (BD Stemflow hMSC Analysis Kit, BD Biosciences). Related isotype antibodies were used as control. The analysis was performed using FACSCanto II (BD Biosciences) and FlowJo software (FlowJo LLC).

ZIKV Infection of hMSC

hMSC were infected at a multiplicity of infection (MOI) of 10, with an adsorption period of 2 h at 37 °C in T75 culture flasks. Subsequently, the inoculum was replaced by fresh DMEM supplemented with penicillin (20 units/mL), streptomycin (20 mg/mL), and 10% (*v*/*v*) heat-inactivated fetal bovine serum (all from Gibco, Gaithersburg, MD), and maintained at 37 °C in a 5% CO_2 atmosphere. Two days post-infection, the medium was removed and PBS buffer was added. Then, the

cells were scraped off, pelleted (5 min, 2000 rpm), and washed once again in PBS. Mock-infected cells were used as controls. All experiments were performed in three independent biological replicates for both analytical groups (Fig. 1).

Protein Extraction and Digestion for Mass Spectrometry

hMSC samples, uninfected (control) and ZIKV-infected cells, were suspended in 100 uL of extraction buffer (8 M urea, 50 mM ammonium bicarbonate, 50 mM HEPES, pH 7.5), containing protease inhibitor cocktail plus EDTA (Thermo Scientific, Rockford, IL). The samples were then sonicated 10 min in ultrasonic bath, following 3 cycles (20 s each) using a tip sonicator (intervals of 1 min on ice after each sonication round). After sonication, samples were centrifuged (14,000 rpm, 10 min, 4 °C) and the supernatants were recovered. Protein concentration was determined using BCA assay kit (Thermo Scientific, Rockford, IL). Approximately 100 μg of samples was suspended in digestion buffer (8 M urea, 100 mM tris-HCl pH 8.5) and treated as previously described [4]. The proteins were digested with 2 μg of trypsin (Promega, Madison, WI) by incubation at 37 °C during 16 h. Proteolysis was stopped by adding formic acid to a final concentration of 5%. Samples were centrifuged at 14,000 rpm for 20 min, and the supernatant was collected and stored at − 80 °C.

MudPIT and Mass Spectrometry Analyses

Briefly, the protein digest was analyzed using a modified 7-step MudPIT separation as previously described [14], ranging from 0 to 100% buffer C. Peptides eluting from the microcapillary column were electrosprayed directly into an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The search was made using ProLuCID algorithm [15] against human and Zika reviewed proteins list from UniProt (downloaded in August 22, 2017). ProLuCID results were assembled and filtered using the DTASelect program [16] resulting in a data set with a false discovery rate of 1% for protein. The MudPIT and mass spectrometry analysis are fully described in Supplementary File 1.

Spectrum raw files were extracted into MS1 and MS2 files using RawConverter [17]. To estimate peptide probabilities and FDRs accurately, we used a target/decoy database containing the reversed sequences of all the proteins appended to the target database. Tandem mass spectra were matched to sequences using the ProLuCID algorithm with 50 ppm peptide mass tolerance for precursor ions and 400 ppm for fragment ions. The search space included all fully and half-tryptic peptide candidates that fell within the mass tolerance window with no miscleavage constraints. Carbamidomethylation (+ 57.02146 Da) of cysteine was considered as a static modification. The validity of peptide/spectrum matches (PSMs) was assessed in DTASelect using two SEQUEST-defined parameters, the cross-correlation score (XCorr), and normalized difference in cross-correlation scores (DeltaCN). The search results were grouped by charge state (+ 1, + 2, + 3, and greater than + 3) and tryptic status (fully tryptic, half-tryptic, and nontryptic), resulting in 12 distinct subgroups. In each of these subgroups, the distribution of Xcorr, DeltaCN, and DeltaMass values for targeted and decoy database PSMs was obtained, and then the targeted and decoy subsets were separated by quadratic discriminant analysis. We performed label-free quantitative analysis using Census through IP2 (Integrated Proteomics Applications, Inc., http://www.integratedproteomics.com) [18]. First, the elemental compositions and corresponding isotopic distributions for each peptide were calculated, and this information was then used to determine the appropriate m/z range from which to extract ion intensities, which included all isotopes with greater than 5% of the calculated isotope cluster base peak abundance. MS1 files were used to generate chromatograms from the m/z range surrounding precursor peptides.

We grouped biological replicates of each sample (Fig. 1) to determine the protein level. Census used protein identification results from DTASelect2 and generated a reconstructed MS1-based chromatogram for each identified peptide. When peptides are not identified in all the relevant samples, Census searched missing peptides using accurate precursor mass, retention time, and charge states and retrieve them and build their chromatograms. We generated peptide ratios by randomly choosing pairs of peptide intensities from the three biological replicates for the two different samples. The Grubbs test (p value < 0.01) was then applied to remove outlier peptides. To increase accuracy for finding peptide precursors, we calculated Pearson product-moment correlation coefficient comparing theoretical and experimental isotope distributions to minimize false peak detection. When peaks are not detected, we calculated background noise to assign small values to peptides.

For all further bioinformatics analysis, all proteins with negative fold change and presenting $p < 0.05$ (ratio p value in Supplementary Table 2) or that were uniquely identified in control condition were considered downregulated and all proteins with positive fold change and presenting $p < 0.05$ or that were uniquely identified in infected hMSC were considered up-regulated. The p value ($p < 0.05$) was calculated based on the normal distribution of the observed protein ratios by using Census software.

Pathway Mapping and Functional Annotation of the Differential Proteome

The FASTA files with identified protein sequences (418 up and 867 down) were given, separately, as input to Blast2GO tool (version 5.1.12) (http://www.blast2go.com) for BLAST

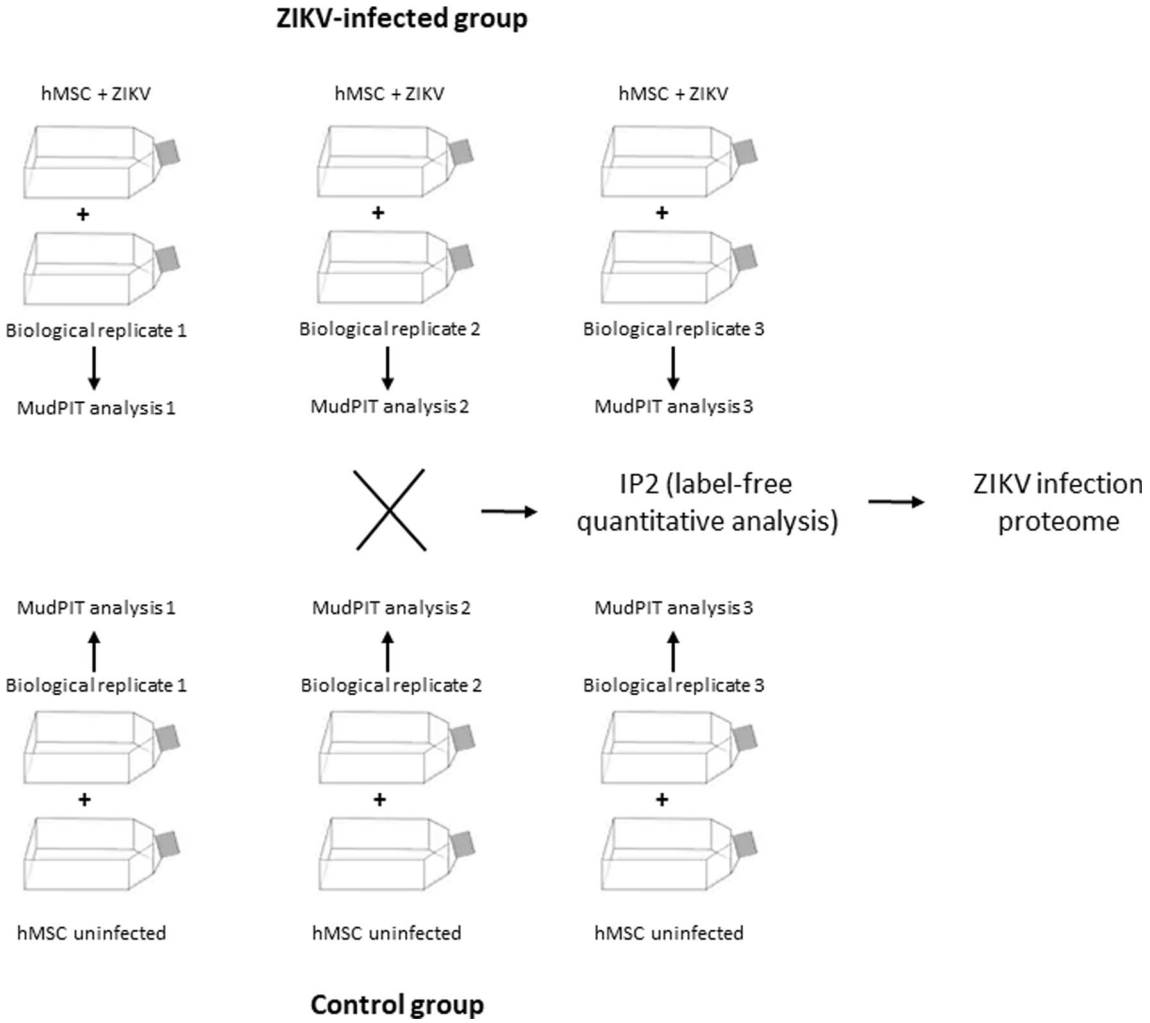

Fig. 1 Experimental design used to identify the differential proteome of ZIKV infection on hMSC

annotation [19]. As part of the Blast2GO analysis, proteins were assigned with an EC (Enzyme Code) number and mapped to the relevant Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways.

The Panther database (http://www.pantherdb.org) was used to categorize the identified proteins in different levels: biological process, molecular function, and cellular component [20], according to Gene Ontology (GO) terms (http://www.geneontology.org), an explicit evolutionary framework to infer annotation of proteins from a broad set of genomes from experimental annotations in a semi-automated manner.

Correlation and Assignment of Identified Proteins to Brain Diseases and Interactome Analysis

To investigate which differentially expressed proteins identified were related to previously described clinical phenotypes and other brain pathologies, comparisons were made between identified proteins and those related to the pathogenesis of human brain diseases. Accordingly, the MalaCards database of human diseases [21] was used, selecting seven clinical phenotypes previously associated to ZIKV infection and 23 others brain diseases (Supplementary Table 1) notoriously known for comparison [22]. The search was made manually for each protein identified against all proteins correlated to those selected diseases.

Further, a protein-protein network analysis of the proteins assigned specifically to those brain diseases was also performed submitting the corresponding protein IDs to the STRING software (version 10.5) (http://stringdb.org/) [23]. For each protein-protein association stored in STRING, a score is provided. These scores (i.e., the "edge weights" in each network) represent confidence scores and are scaled between 0 and 1. Proteins were represented with nodes and the interactions with continuous lines showing direct interactions (physical), all being numerically quantified (node score). The node scores indicate the estimated likelihood that a given interaction is biologically meaningful, specific, and reproducible, given the supporting evidence. For each interaction, the

supporting evidence is divided into one or more "evidence channels," depending on the origin and type of the evidence. All the edges were supported by at least a reference from the literature or from canonical information stored in the STRING database. Cytoscape software (version 3.6.1) [24] was applied to visualize the protein-protein interaction relationship network. Centiscape 2.2 application in Cytoscape was used to calculate the degree related with the predicted regulatory relevance of each node [25].

Results

Differential Proteome Overview of hMSC Infected with ZIKV

A total of 2518 proteins were identified in the hMSC proteome when infected with ZIKV (Supplementary Table 2). Among these proteins, 145 were considered differentially expressed ($p < 0.05$), with 27 upregulated and 118 downregulated, compared to control cells. Moreover, 295 and 635 proteins were uniquely identified in infected hMSC and in control cells, respectively.

The top 20 most affected pathways, in number of proteins, are presented in Fig. 2a, most of them relate to energy metabolism and nucleotide pathways. Proteins related to metabolism of purines, amino acids (glycine, serine, and threonine), and lipids (phosphatidylinositol) as well as signaling pathways, such as phosphoinositide 3 kinase (PI3K) were downregulated upon infection. Proteins were assigned according to gene ontology (GO) levels (cellular component, molecular function, biological process) based on their expression (Fig. 2b–d). Proteins involved in cellular and metabolic processes and related to binding and catalytic activities were mostly downregulated. For proteins categorized as cellular components (Fig. 2c), the majority of the proteins were assigned to organelles, macromolecular complexes or membranes.

Brain Diseases Protein Correlation

All proteins identified in the proteome were manually checked in the MalaCards database, to correlate protein molecular annotation to ZIKV pathological consequences and to other well-known brain diseases. The highest number of proteins assigned to pathological phenotypes already associated to ZIKV was found related to microcephaly with 13 protein references identified, followed by congenital central nervous system diseases and epilepsy (Fig. 3a; Supplementary Table 3). Proteins related to other brain diseases not yet related to ZIKV infection were found. These proteins in order of numbers were related to amyotrophic lateral sclerosis (ALS), with 23 protein references, followed by schizophrenia, Alzheimer, Parkinson diseases, and autism (Fig. 3b). All proteins associated with brain diseases were used in a protein-protein interaction network analysis (Fig. 3c). The interactome showed a close association between proteins related to microcephaly and schizophrenia, ALS, Alzheimer and Parkinson diseases, and dementia.

We also attributed degrees to proteins in the interactome (Supplementary Table 3). The biological meaning of the degree is the potential regulatory relevance of each node. Proteins with higher degree interact with multiple proteins, thus suggesting a central regulatory role, being considered as a protein hub, such as PAFAH1B1, NDE1, CASP3, and APP. For instance, CASP3, corresponding to caspase 3 protein involved in apoptosis, is associated with congenital CNS disease, Alzheimer, and ALS. This protein interacts with 10 other proteins, most already described in ALS (Fig. 3c). In the same way, PAFAH1B1, corresponding to platelet-activating factor acetylhydrolase, associated with schizophrenia, interacts with proteins previously associated with microcephaly and schizophrenia.

Discussion

While different aspects of ZIKV pathology are undergoing scientific study, the molecular process of infection remains poorly characterized. A global proteomic view of hMSC ZIKV infection points to metabolic pathways being most impacted and describes new molecular pathways and potential mechanisms to be investigated. By considering the entire proteomic dataset, 101 different pathways were found affected by ZIKV infection. In other cells, it has been also shown that some pathways are disturbed by ZIKV infection [26–28], suggesting a potential cell reprograming towards nucleotide synthesis, energy metabolism, signaling pathways, among others [7, 26], similarly to the pattern found in our results.

Interestingly, proteins from signaling pathways including PI3K and mTOR (Supplementary Fig. 1 and Supplementary Fig. 2) were found inhibited by ZIKV infection, which was previously shown to culminate in alterations in autophagy, neurogenesis, and immune response [29, 30]. Brain malformations, such as microcephaly, are associated with alterations in PI3K/Akt and mTOR pathways [29], leading to increase viral proliferation due to inhibition of PI3K, a classical pathway associated to cellular proliferation [30]. Moreover, PI3K/Akt and mTOR are closely associated to several other pathways that maintain brain homeostasis. In addition, both might involve cellular dysfunction, inflammatory response, impairment in redox status with consequent nitrosative/oxidative stress, and metabolic alterations. In this sense, PI3K is a crucial signal to triggering inflammatory response, through NFκB transcriptional activity in neural cells [31–33]. These pathways might coordinate eukaryotic cell growth and metabolism, including nutrients and growth

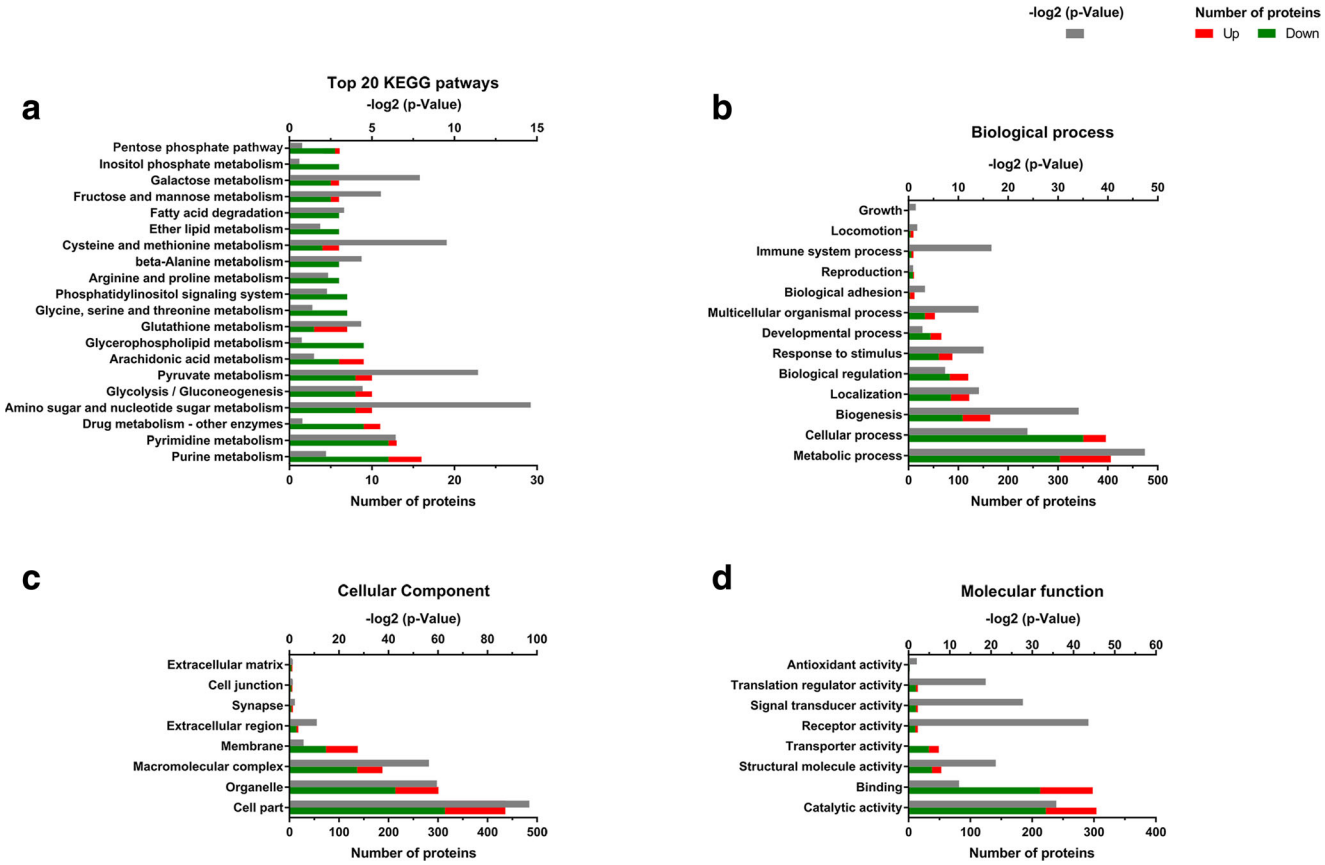

Fig. 2 Functional characterization of differential proteome of hMSC infected with ZIKV. (A) Top 20 most impacted KEGG pathways, in number of proteins. Gene ontology assignment of proteins in different levels: (B) biological process, (C) molecular function, and (D) cellular component. For these analyses, all proteins with negative fold change and presenting $p < 0.05$ or that were uniquely identified in control condition were considered downregulated and all proteins with positive fold change and presenting $p < 0.05$ or that were uniquely identified in infected hMSC were considered up-regulated (Supplementary Table 1)

factors [34]. Thus, these signaling pathways could be the key in cellular events linked to microcephaly and the progression of neurological disorders potentially associated with ZIKV.

To ensure optimal environments for their replication and spread, viruses have evolved to alter many host cell pathways [35], especially those related with nucleotide metabolism. It results in increased nucleotide biosynthesis, thus providing substrates for synthesis of purine and pyrimidine for viral replication, [36, 37]. In addition, most viruses evaluated by metabolomics induce alterations in cellular metabolism, including fatty acid synthesis. These modifications of carbon source utilization by infected cells can increase available energy for virus replication and virion production, creating viral replication niches while increase infected cell survival [35], corroborating with KEGG results. In the same way, metabolic alterations induced by ZIKV infection culminate in the overexpression or accumulation of some metabolites, including lipids [29]. As observed in the phosphatidylinositol signaling system (Supplementary Fig. 3), proteins from this pathway were found altered with potential accumulation of two lipids, PI (1-phosphatidyl-D-myo-inositol) and PIP2 (phosphatidylinositol-4,5-bisphosphate), already described as possible biomarkers of ZIKV-infected patients [29]. Therefore, ZIKV infection has induced alterations in different signaling pathways, which have culminated with the overexpression of some metabolites, including above-reported lipids.

Taken together, our data and the above-described data, these effects can be noticed not only in a wider view (metabolic pathways), but also in specific gene/protein identifications. For example, we have identified in hMSC 88 proteins corresponding to 69 genes previously identified with altered expression due to ZIKV infection in four different cell types [26]. Comparing hMSC results with a neurosphere dataset [7], there are 219 shared protein identifications. Nevertheless, our proteome analysis presents a set of novel proteins, molecular processes, and pathways affected by ZIKV infection in hMSC. Also, the majority of the studies until now have identified host-virus interactions by identifying genes affected through mRNA transcriptional profiling. However, proteomic data is closer to the physiological processes to better understand the biological phenomena and mechanisms [4, 38]. Furthermore, several studies note the poor correlation between mRNA and

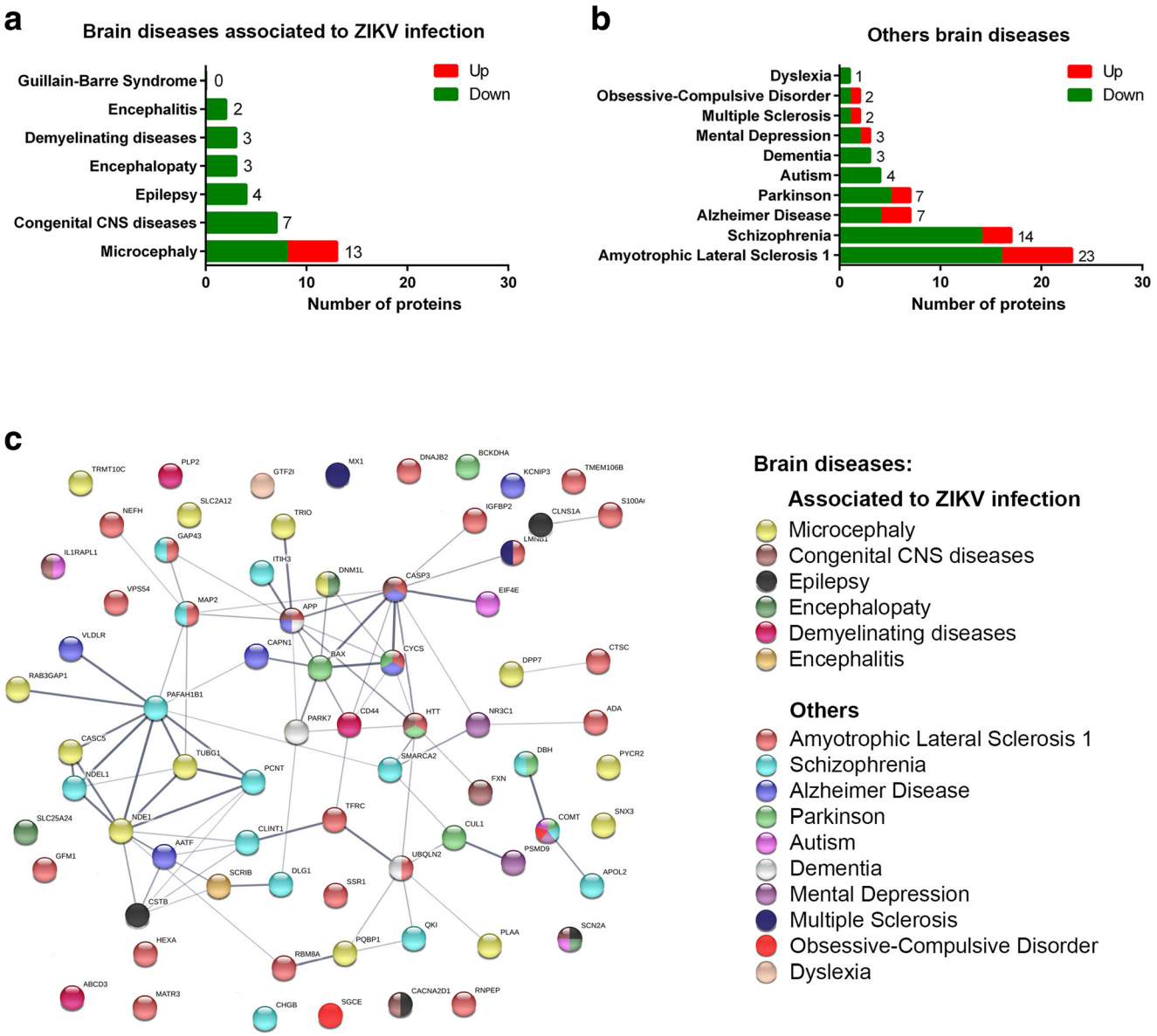

Fig. 3 ZIKV differentially expressed proteins (DEPs) associated with human brain diseases. Number of proteins assigned to brain diseases (A) previously associated to ZIKV infection or (B) assigned to other brain diseases. (C) Protein-protein interaction network of proteins showed in A and B. Proteins were represented by nodes and the interactions with continuous lines, where thicker lines represent stronger interaction. Proteins presented here were considered statistically ($p > 0.05$) differentially regulated (up- or downregulated or unique)

protein levels [39–41] suggesting that to understand the molecular mechanisms of the host-virus interaction requires measurements at the proteome level.

Since ZIKV effects were shown to be mainly promoted in the brain and have presented clear brain clinical phenotypes, like microcephaly [1, 22, 29], our results point to a potential link to other unexpected brain diseases according to protein neuropathological annotation. The association of diseases with metabolic routes, genes, and signaling pathways is a central theme of present-day research scrutiny and it is very helpful for more meaningful conclusions and selection of molecular targets for additional studies, including biomarkers, and potential therapeutic strategies [21, 42]. After searching for correlations between the proteins identified in our study against 30 different brain disorders, 74 different proteins were found implicated in 16 neuropathologies (six out of seven phenotypes already associated to ZIKV infection, as expected, and 10 out of 23 to other brain diseases). Furthermore, although with different clinical symptomatology, the brain disorders associated to ZIKV infection described here share some cellular and molecular mechanisms; thus, the proteomic analyses might improve the understanding of pathomechanisms of brain diseases. A protein interaction network was built only with these proteins to improve the selection of potential

candidates for future deep surveys, since they are already known in the context of brain pathologies. Moreover, the identification of specific biomarkers/targets not only can increase the understanding of the molecular basis of ZIKV pathology but also may link to potential new clinical consequences, unknown yet, since most of the individuals infected in the outbreak are under surveillance. Proteins like CASP3, APP, NDE1, CYCS, and PAFAH1B1 are central nodes interacting with multiple proteins being potential targets to be further analyzed. In line with this, NDE1 protein, which presented higher degree in our network, is related to cognitive disability, a crucial event in Alzheimer's disease, as well as APP and CASP3 proteins, which are associated with neuronal death in the same condition [43]. In addition, MAP2 stabilizes dendritic shape during neuron development/survival and CYCS regulates mitochondrial functionality, including apoptosis [44]. In the interactome, MAP2 is a node linking two major hubs: CASP3 and PAFAH1B1. CASP3 is the predominant caspase involved in the cleavage of amyloid-beta 4A precursor protein, which is associated with neuronal death in Alzheimer's disease [45]. In the same way, PAFAH1B1, which regulates the amount of platelet-activating factor (PAF) in the brain, a protein involved in neuronal migration, is essential for normal brain development and function [46]. Thus, we proposed the association between specific proteins altered in our experimental model and brain disorders, independent of microcephaly. These proteins are also strongly linked to cellular components, such as synaptic and membrane functions (Fig. 2C) [47]. Moreover, PI3K/Akt regulates membrane trafficking of several neural proteins, including glutamate transporters that are involved in the pathogenesis of several brain diseases [48].

In addition, a new observation of monitored subjects is important to check any new potential clinical phenotype, as it was detected in our molecular data. It is surprising the increased number of proteins found implicated in neuropathologies not yet related to ZIKV infection. Anyway, the cloudy panel of ZIKV infection and pathology can lead to speculation based on molecular data on potential brain disorders usually happening later in life. For instance, ZIKV alters the DNA methylome of multiple cells at genes that have been implicated in the pathogenesis of a number of neuropathologies, like schizophrenia [49]. Accordingly, this disease was the second most prominent in number of correlated proteins in our analysis. It can certainly lead to preventive assumption of all these potential phenotypes and improve diagnosis and therapeutics of individuals that are still suffering with undergoing neurological impairment due to ZIKV.

The understanding of fundamental mechanisms of ZIKV pathogenesis in hMSC could help future strategies to prevent ZIKV infections and its consequent negative effects [10, 50]. Our report is adding a substantial amount of new of protein information about the impact of ZIKV on the molecular pathways of hMSC that are disrupted and are related to brain diseases that will be very helpful in increasing our understanding of ZIKV infection and associated diseases. This information can be used in preventive clinical surveys for new pathological phenotypes helping in treatment and diminishing the associated damage of ZIKV neurological disorders.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval The study was approved by the institutional research ethics committee of Hospital de Clínicas de Porto Alegre (Federal University of Rio Grande do Sul) under protocol # 2018-0059.

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