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COMPARATIVE PROTEOMIC PROFILING REVEALS ABERRANT CELL PROLIFERATION IN THE BRAIN OF EMBRYONIC TS1CJE, A MOUSE MODEL OF DOWN SYNDROME

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Abstract—To identify molecular candidates involved in brain disabilities of Ts1Cje, a mouse model of Down syndrome (DS), we performed comparative proteomic analyses. Proteins extracted from the brains of postnatal wild-type (WT) and Ts1Cje mice were analyzed by two-dimensional gel electrophoresis (2-DE). No differences were detected in the proteins expressed in the whole brain between WT and Ts1Cje mice at postnatal day 0 and 3 months of age. Five spots with differential expression in the brains of Ts1Cje mice were detected by 2-DE of brain proteins from WT and Ts1Cje embryos at embryonic day 14.5 (E14.5). These differentially expressed proteins in Ts1Cje embryos were identified as calcyclin-binding protein (CACYBP), nucleoside diphosphate kinase-B (NDPK-B), transketolase (TK), pyruvate kinase (PK), and 60S acidic ribosomal protein P0 (RPLP0) by peptide mass fingerprinting. CACYBP and NDPK-B were involved in cell proliferation, whereas TK and PK were associated with energy metabolism. Experiments on cell proliferation, an *in vivo* bromodeoxyuridine (BrdU)-labeling experiment, and immunohistochemical analysis for phospho-histone H3 (an M-phase marker) demonstrated increased numbers of BrdU-positive and M-phase cells in the ganglionic eminence. Our findings suggest that the dysregulated expression of proteins demonstrated by comparative proteomic analysis could be a factor in increased cell proliferation, which may be associated with abnormalities in DS brain during embryonic life.

Key words: Down syndrome, brain development, comparative proteomics, cell proliferation.

INTRODUCTION

Down syndrome (DS), which is caused by triplication of human chromosome 21 (HSA21), is the most frequent aneuploidy that involves mental and developmental retardations (Epstein, 2001). Neuropathology, such as a small brain, reduced number of neurons, lower density of dendritic spine, impaired plasticity, and early onset of Alzheimer's disease-like neurodegeneration, is seen in most patients with DS (Epstein, 2001; Antonarakis et al., 2004). The molecules and pathomechanisms associated with the symptoms of DS remain unclear.

Several mouse models of DS have been used to investigate the pathomechanisms of DS symptoms *in vivo* (Das and Reeves, 2011; Edgin et al., 2012; Haydar and Reeves, 2012; Yamakawa, 2012). Because the distal end of mouse chromosome 16 (MMU16) is syntenic with the large part of HSA21, the mouse models of DS, such as the Ts65Dn, Ts1Cje, Tc1, Ts1Rhr and Ts1Yey strains carry full or segmental trisomy of MMU16 (Davisson et al., 1990; Sago et al., 1998; Olson et al., 2004; O'Doherty et al., 2005; Yu et al., 2010). In contrast, Ts2Yey mice carry an extra MMU10 segment that is homologous to HSA21, and Ts1Yah and Ts3Yey mice have MMU17 segments that are also homologous to HSA21 as a trisomic region (Pereira et al., 2009; Yu et al., 2010). Ts1Cje mice have a segmental trisomy of MMU16, which spans from Cu/Zn-superoxide dismutase (*Sod1*) to *Znf295*, although the third copy of *Sod1* is inactivated by insertion of a neomycin cassette (Sago et al., 1998). It has been shown that overexpression of genes in the trisomic region of Ts1Cje mice occurs in a gene dosage-dependent manner in the neonatal period (Amano et al., 2004). Adult Ts1Cje mice show some DS-related abnormalities, such as craniofacial alterations (Richtsmeier et al., 2002) and spatial learning deficits

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Abbreviations: 2-DE, two-dimensional gel electrophoresis; BrdU, bromodeoxyuridine; CACYBP, calcyclin-binding protein; DAB, 3,3′-diaminobenzidine; DAVID, Database for Annotation, Visualization, and Integrated Discovery; DS, Down syndrome; E14.5, embryonic day 14.5; GE, ganglionic eminence; HSA21, human chromosome 21; IgG, immunoglobulin G; MALDI-TOF, matrix-associated laser desorption/ionization time-of-flight; MGE, medial ganglionic eminence; MMU16, mouse chromosome 16; MS, mass spectrometry; NDPK-B, nucleoside diphosphate kinase-B; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PH3, phospho-histone H3; PK, pyruvate kinase; qRT-PCR, quantitative reversed transcription-PCR; *Rcan1*, regulator of calcineurin 1; RPLP0, 60S acidic ribosomal protein P0; SDS, sodium dodecyl sulfate; *Sod1*, Cu/Zn-superoxide dismutase 1; TBS-T, Tris-buffered saline containing 0.05% Tween-20; TK, transketolase; WT, wild-type.

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(Sago et al., 1998). In addition, reduction of hippocampal long-term potentiation has been observed in adult Ts1Cje mice (Siarey et al., 2005; Belichenko et al., 2007). We have observed increased oxidative stress in the brains of adult Ts1Cje mice (Shukkur et al., 2006; Ishihara et al., 2009) and decreased adult and embryonic neurogenesis in the brains of Ts1Cje mice (Ishihara et al., 2010). Thus, Ts1Cje mice show various abnormal phenotypes in both prenatal and postnatal periods. Identification of molecules responsible for DS abnormalities is important for understanding their pathogenesis.

Proteomic analysis, which involves large-scale profiling of proteins, is a powerful tool for the detection of differentially regulated expression of molecules. In addition, post-translational modified proteins can be quantified in this comprehensive analysis. In the present study, we performed comparative proteomic profiling to identify and compare differentially expressed proteins in the brains of Ts1Cje and wild-type (WT) mice during adult and embryonic periods.

EXPERIMENTAL PROCEDURES

Mice

Ts1Cje mice were maintained by crossing carrier males with C57BL/6J females under conventional housing conditions. Genotyping of Ts1Cje mice was performed by polymerase chain reaction (PCR) using multiplex primers for the neomycin resistance gene and the ionotropic glutamate receptor kainate 1 (internal control), as described previously (Amano et al., 2004). Mouse embryos were derived from crosses of Ts1Cje males and C57BL/6J females, and the day of vaginal plug was considered to be embryonic day 0.5 (E0.5). All mice were housed fewer than five per cage under a 12-h light–dark cycle and with *ad libitum* access to food and water. All experimental procedures were performed in accordance with the guidelines of the Animal Experiments Committee of Kyoto Pharmaceutical University.

Comparative proteomic analysis

For analysis of the adult brain ($n = 5$ in each genotype), mice were anesthetized (Avertin, 2.5%, 0.3 ml/25 g body weight i.p.) and perfused transcardially with saline. Brains were immediately isolated, and the hippocampus, cerebral cortex, and striatum were separated in ice-cold saline under a stereomicroscope. For analysis of embryonic ($n = 5$ in each genotype) and neonatal mice ($n = 5$ in each genotype), the brains from pups were dissected at embryonic day 14.5 (E14.5) or P0. These brains were stored at −80 °C until analyzed. Comparative proteomic analysis based on two-dimensional gel electrophoresis (2-DE) was performed as described previously (Ishihara et al., 2005). Two-dimensional (2D) gel imaging and Proteomweaver™ 2D analysis software (Bio-Rad Laboratories, Redmond, WA, USA) were used for quantification of protein spots, gel-to-gel matching, and identification of differences in spot intensity. The intensity of each spot in a gel was quantified by calculating the spot volume after normalization of the image by using the total spot volume normalization method and multiplying by the total area of all spots. Differences in the intensities of matched individual protein spots were analyzed, and statistical significance was determined using Student's *t*-tests. For identification of proteins in a gel, peptide mass fingerprinting was performed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (Voyager-DE RP; Per-Septive Biosystems Inc., Framingham, MA, USA), as described previously (Ishihara et al., 2005). Proteins were identified using MS-fit (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msfitstandard). The search parameters were set as follows: mass tolerance, ±1 Da; number of missed cleavage sites, up to 2; the cysteine residue was modified to carbamidomethyl-Cys; and the peptide ion was $[M+H]^+$.

Bioinformatics analyses

Functional annotation analysis of proteins with differential expression in the brains of Ts1Cje mice was performed using Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.7 (http://david.abcc.ncifcrf.gov/home.jsp; Dennis et al., 2003; Huang et al., 2009). A 'genelist' was compiled of all target genes differentially expressed in Ts1Cje mice when compared with WT mice and uploaded to the DAVID web interface using the MGI ID against the whole mouse genome.

Immunoblot analysis

Embryonic brains (E14.5) or cerebral cortices (3 months of age) from WT and Ts1Cje mice were homogenized in ice-cold radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] supplemented with a complete protease inhibitor cocktail tablet (Roche Diagnostics, Indianapolis, IN, USA) and phosphatase inhibitors (Wako Pure Chemical Industries Ltd., Osaka, Japan). Protein extract was obtained after 10 min of centrifugation at 15,000 rpm, 4 °C. The protein concentration was determined by the Bradford method. A 37.5-μg sample of protein was denatured and subjected to SDS–12.5% polyacrylamide gel electrophoresis (PAGE) and then transferred onto nitrocellulose membranes. After blocking the membranes with 3.5% skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBS-T), the membranes were incubated with antibodies against nucleoside diphosphate kinase-B (NDPK-B) (diluted at 1:2500 with 3.5% skim milk/TBS-T; Abcam, Cambridge, MA, USA), calcyclin-binding protein (CACYBP) (1:1000 with 3.5% skim milk/TBS-T; Cell Signaling Technology, Danvers, MA, USA), transketolase (TK) (1:1000 with 3.5% skim milk/TBS-T; Cell Signaling Technology), pyruvate kinase (PK) M1/2 (1:1000 with 3.5% skim milk/TBS-T; Cell Signaling Technology), 60S acidic ribosomal protein P0 (RPLP0) (1:2000 with 3.5% skim milk/TBS-T; Abcam), and β-actin (1:5000 with 3.5% skim milk/TBS-T, Sigma–Aldrich, St. Louis, MO, USA) for 1 h at room temperature. After washing with TBS-T,