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An extra human chromosome 21 reduces mlc-2a expression in chimeric mice and Down syndrome☆

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Abstract

An extra copy of human chromosome 21 (Chr 21) causes Down syndrome (DS), which is characterized by mental retardation and congenital heart disease (CHD). Chimeric mice containing Chr 21 also exhibit phenotypic traits of DS including CHD. In this study, to identify genes contributing to DS phenotypes, we compared the overall protein expression patterns in hearts of Chr 21 chimeras and wild type mice by two-dimensional electrophoresis. The endogenous mouse atrial specific isoform of myosin light chain-2 (mlc-2a) protein was remarkably downregulated in the hearts of chimeric mice. We also confirmed that the human MLC-2A protein level was significantly lower in a human DS neonate heart, as compared to that of a normal control. Since mouse mlc-2a is involved in heart morphogenesis, our data suggest that the downregulation of this gene plays a crucial role in the CHD observed in DS. The dosage imbalance of Chr 21 has a trans-acting effect which lowers the expression of other genes encoded elsewhere in the genome.

Keywords: Human chromosome 21; Down syndrome; Congenital heart disease; Two-dimensional electrophoresis; Atrial myosin light chain-2; Chimeric mouse containing human chromosome 21; Dosage imbalance

Down syndrome (DS) is a congenital disease caused by trisomy of human chromosome 21 (Chr 21) and is characterized by mental retardation and a variety of developmental anomalies including facial dysmorphology, congenital heart defects (CHD), infertility, immunodeficiencies, and an increased incidence of leukemia [1]. More than 50% of DS patients have CHD; most commonly, endocardial cushion defects, atrioventricular valve malformations, atrial septal defects, and ventricular septal defects. Thus, cardiac problems are leading causes of deaths in infancy and early childhood of DS individuals [2]. Although it has been suggested that overexpression of a gene(s) or gene cluster on Chr 21 plays a key role in bringing about changes of DS phenotypes, little is known about the molecular mechanisms underlying CHD and mental retardation associated with DS [3].

☆ *Abbreviations:* DS, Down syndrome; Chr 21, human chromosome 21; CHD, congenital heart disease; 2-DE, two-dimensional electrophoresis; Chr 21 chimeric mouse, chimeric mouse containing an extra copy of human chromosome 21; wild type, regular chimeric mouse derived form normal ES cells; ES[Chr 21], ES cells containing an extra copy of human chromosome 21; control ES, normal ES cells; mlc-2a, atrial specific isoform of myosin light chain-2; SOD1, superoxide dismutase [Cu–Zn].

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Recent studies of DS individuals with rare partial duplications of Chr 21 suggested that the candidate region responsible for CHD in DS, DS-CHD, is located somewhere within 21q22.13, proximal to 21q22.3. A candidate gene, Down syndrome cell adhesion molecule (DSCAM), has been identified within this region [4,5]. However, the resolution of this approach is limited by the substantial phenotypic variability among DS individuals. Thus, it is difficult to rationalize a model wherein only a single gene contributes to the DS phenotype.

Animal models are useful for understanding the molecular mechanism of the phenotype–genotype correlation of DS [6]. Because wild type and model animals share the same genetic background, animal models are not affected by individual differences and environmental factors. It is also easy to identify transgene-associated changes among model animals derived from the same parental strains.

In our previous study, chimeric mice were produced using ES cell clones containing an almost intact copy of Chr 21 (Chr 21 chimeric mice) [7–10]. These mice demonstrated specific developmental anomalies including learning or behavioral impairments and cardiac anomalies, similar to the clinical manifestations of DS. Pathological analysis of hearts from chimeric fetus' that exhibited more than 50% retention of Chr 21, showed a variety of cardiovascular anomalies, such as double outlet right ventricle, riding aorta, ventricular septal defects, morphologenic anomalies of the pulmonary artery, atrioventricular canal malformation, and myocardial layers of the heart ventricles. These anomalies were not observed in several mice with segmental trisomy of mouse chromosome 16 [11,12]. We also reported that the introduction of an extra Chr 21 into ES cells may lead to the disturbance of cardiogenesis, as observed during in vitro differentiation into cardiac tissue [13]. Thus, the introduction of Chr 21 resulted in a gene dosage imbalance that led to the manifestation of a DS phenotype.

Here, proteomic analysis with two-dimensional electrophoresis (2-DE) was performed to identify candidate gene products responsible for DS phenotypes and to examine the expression differences between wild type and Chr 21 chimeric mice. The advantage of this experimental strategy is that it can directly visualize the overall dosage imbalance of gene products caused by trisomy. Previously, 2-DE analysis was performed on brain tissues of human DS specimens and normal controls [14]. Lowered snap-25 protein levels were observed in DS brains, suggesting impaired synaptogenesis or neuronal loss. However, to our knowledge, no study has ever reported results from protein analysis of human DS heart tissue or animal models.

In this study, we demonstrated that the endogenous atrial specific isoform of myosin light chain-2 (mlc-2a) was post-transcriptionally downregulated in Chr 21 chimeric mice and in a human DS patient, suggesting that downregulation of a non-Chr 21 gene by the extra Chr 21 may have implications in DS phenotypes. We have also validated the potential use of protein analysis and a proteomic approach for the identification of gene products causing dosage imbalance of trisomy 21 in our DS model.

Materials and methods

Tissue samples, ES cells. Chr 21 chimeric mice, c10-4, c10-5, c10-7, and c10-9, and corresponding regular chimeric mice (wild types), wild 1 and wild 2, were generated as described previously [7]. These four Chr 21 chimeric mice showed higher than 90% chimerism in coat color. The retention rate of Chr 21 was more than 50% in the heart of each chimera. In this analysis hippocampus tissue samples were obtained from c10-7 and c10-9 chimeric mice, which exhibited behavioral impairment in open-field tests. Age matched human neonate tissue samples of DS and normal specimens were obtained with informed consent. ES[Chr 21] and control ES used to produce chimeric mice were generated as described previously [7,13].

2-DE analysis. Protein extraction and 2-DE analysis were performed following standard 2D-PAGE protocol with minor modifications [15–17]. Tissues were homogenized in four volumes of lysis buffer (7 M urea, 2 M thiourea, 1.5% w/v Triton X-100, 0.5% w/v CHAPS, 0.5% v/v pharmalyte, 10 mg/ml DTT, 5 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM PMSF) and disrupted by ultrasonication. The supernatant of the tissue lysate was obtained by centrifugation and the protein concentration was estimated by dot blot staining. In the first-dimension of isoelectric focusing (IEF), 4.5 μl aliquots of protein extract was applied near the cathode wick on each 17 cm immobilized pH-gradient Readystrip gels, pH 3–6, 4–7, 5–8, and 7–10, and run using the PROTEAN IEF cell system (Bio-Rad, Hercules, CA). In the second-dimension of SDS–PAGE, equilibrated Readystrip gels were placed on top of the polyacrylamide gel (12%, $18 \times 16.5 \times 0.09$ cm) and run vertically with SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1% w/v SDS). After electrophoresis, proteins were detected using a silver-staining reagent kit or Quick CBB staining reagent kit (Wako). Protein spots were quantified using the PDQuest 6.2 software (Bio-Rad, Hercules, CA).

Identification of proteins. Protein spots on the CBB-stained 2-DE gel were excised and digested with lysylendopeptidase AP-1 or trypsin (Wako Pure Chemical, Japan) in 100 mM Tris–HCl (pH 9) overnight at 37 °C with shaking. The digested peptides were extracted with 0.1% TFA and purified by a HPLC system (Model 172, Applied Biosystems) with a C8 column (1×100 mm, RP-300, Applied Biosystems). The amino acid sequences of the recovered peptides were determined with a pulse liquid phase sequencer (Model 492 cLC, Applied Biosystems). The Swiss-Plot database was used for homology searches [18].

Western blotting. After transfer blotting, detection was performed using the ECL detection system (Amersham Pharmacia) following manufacturer's instructions. Ponceau S staining was carried out to determine whether each blot contained the same amount of protein.

Monoclonal anti-myosin (light chains 20k) (Sigma, 1:500 dilution in PBS-T) was used as primary antibody while the peroxidase-conjugated Affinipure goat anti-mouse IgM (Jackson Immune Research, 1:5000 dilution in TBST) was used as secondary antibody.

Real time RT-PCR

The cDNA was prepared from whole hearts of control mice, #1 and #2 (F1: C57BL/6×CBA), and Chr 21 chimeric mice as previously described [7]. Quantitation of mRNA levels for mouse mlc-2a (Gen-

Bank Accession No. AF232920) was performed by TaqMan assay using ABI PRISM 7900 Sequence Detection System, following manufacturer's instructions (PE Applied Biosystems, Foster City, CA) [19,20]. The fluorescent labeled TaqMan probe and non-labeled primers that were designed with Primer Express 1.5 software (Applied Biosystems, Japan) are as follows: 5′-(FAM)-CTGAAGGAGACCAT TCCCAGCTCGGA-(TAMRA)-3′ (designed over the junction of exon 3 and exon 4), 5′-ATGGGATCATCTGCAAATCAGA-3′, 5′-AGCT CTTCCTCCGGAACACTTA-3′. Dilutions of a cDNA sample prepared from whole heart of a control mouse were used to construct a relative standard curve of critical threshold cycle for the *mlc-2a* amplification. The amount of mlc-2a cDNA in each sample was expressed as an *n*-fold difference relative to the control mouse as a calibrator.

Results

2-DE comparison of chimeric mice containing Chr 21 and wild types

To determine the gene dosage effects of an extra copy of Chr 21 in vivo, we compared overall protein expression patterns in whole heart and hippocampus of Chr 21 chimeric (heart, $n = 4$; hippocampus, $n = 2$) and wild type mice (heart, $n = 2$; hippocampus, $n = 2$) by 2-DE analysis. Control wild type mice were produced from normal ES cells. Proteins obtained from chimeric tissues were resolved by 2-DE and were detected by silver staining. Analysis by PDQuest 6.2 software showed that the majority of proteins had normal expression patterns. Differences in dot intensity were observed in only a few spots. Out of 843 protein spots, only one spot was observed to be absent in the wild type mice, but present in hippocampus of the Chr 21 chimeric mice (2/2 cases, Figs. 1A and B). For whole heart, 495 protein spots were observed. Out of these 495 protein spots, one spot had a lesser intensity in Chr 21 chimeric mice (4/4 cases, Figs. 1C and D).

We identified these spots by in situ digestion with lysylendopeptidase, and subsequent isolation of peptides using HPLC, followed by sequencing of purified peptides. Fig. 1G shows the result of HPLC purification and Table 1 shows the amino acid sequences. Using Swiss-Plot, all of the amino acid sequences exhibited 100% homology to known proteins [18]. Spot 1 from heart extract was identified as myosin light chain-2 isoform mlc-2a (mlc-2a), which was downregulated in whole hearts of Chr 21 chimeric mice. On the other hand, spot 2 from hippocampus extracts of chimeric mice was identified as human superoxide dismutase [Cu–Zn] (SOD1). The identified protein names and corresponding spots are shown by arrows in Fig. 1.

To confirm differences in protein expression levels of mouse mlc-2a, proteins obtained from whole heart of each chimeric mice (Chr21 chimeric mice, $n = 2$; wild types, $n = 2$) were resolved by 2-DE, and then examined by Western blotting using anti-myosin monoclonal antibody (light chains 20k). Figs. 1E and F show that

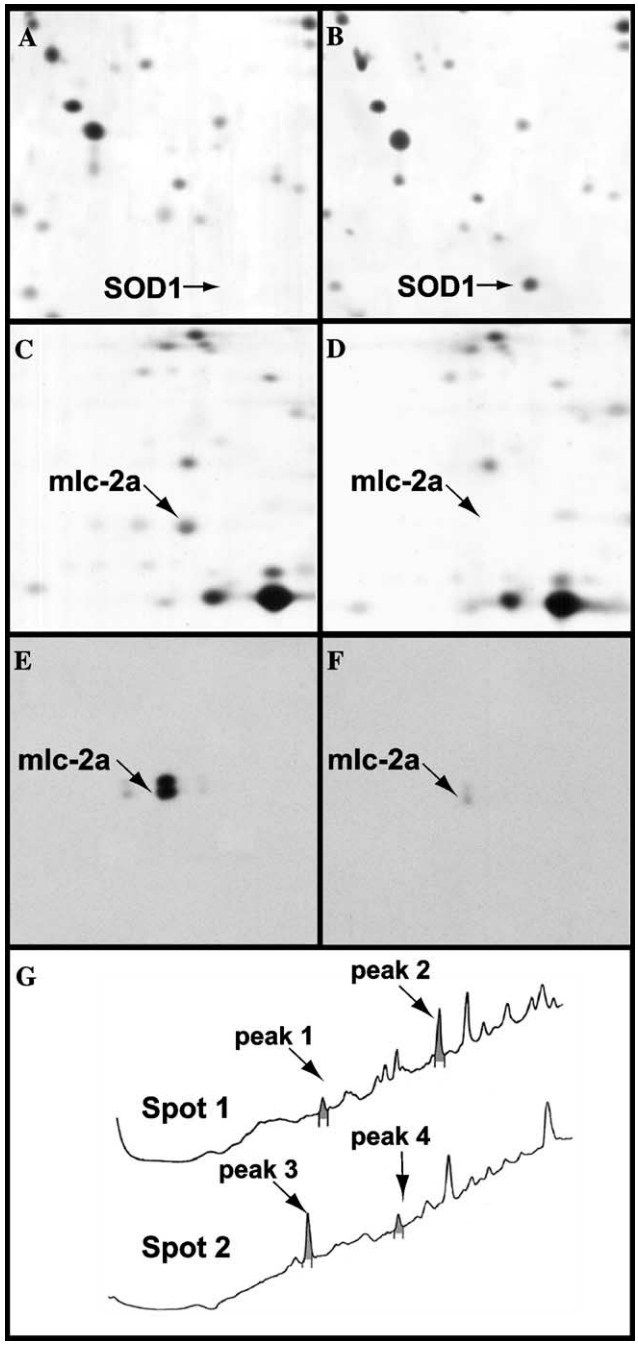

Fig. 1. 2-DE protein expression patterns in Chr 21 chimeric mice and wild type tissues, and peptide purification by HPLC. (A)–(F) show close up areas of 2-D gel protein patterns in tissues of Chr 21 chimeric and wild type mice. Hippocampus proteins were separated by 2-DE and detected by silver staining (A) and (B). Proteins from whole heart were separated by 2-DE and detected by silver staining (C) and (D) or Western blotting with monoclonal anti-myosin light chain (20k) (E) and (F). (A), (C), and (E) were derived from wild type, and (B), (D), and (F) were from Chr 21 chimeric mice. The same volume of tissue extract was applied to each gel, (A)–(B) and (C)–(F). (G) were HPLC-purified peptides from spot 1 and spot 2. The intensity of spot 1 was reduced in whole heart of Chr 21 chimeric mice. Spot 2 was detected in the hippocampus of Chr 21 chimeric mice. All fractions corresponding to peaks were collected manually. The two peptide peaks marked with numerals were analyzed with a protein sequencer.

Table 1
Peptide sequences of the HPLC peaks and corresponding proteins identified by Swiss-Plot

Spot no.	HPLC peaks	Sequences	Swiss-Plot #	Identification protein name
Spot 1	Peak 1 Peak 2	QLLMTQADK ETYSQLGR	Q63977	Myosin light chain-2 isoform MLC-2A
Spot 2	Peak 3 Peak 4	DGPVQGIIN VWGSIK	P08228	Superoxide dismutase [Cu–Zn]

mouse mlc-2a protein is downregulated in Chr 21 chimeric mice (2/2 cases).

Quantitative analysis of mlc-2a protein expression level

To further confirm the difference in protein expression of mouse mlc-2a, we analyzed the protein expression levels in whole hearts of chimeric ($n = 4$) and wild type mice ($n = 2$) quantitatively. Fig. 2A shows the differences in the intensity of spots in whole heart tissue as detected by silver staining using the PDQuest software. In this gel, all Chr 21 chimeric mice exhibited downregulation of mlc-2a.

RNA analysis using real time quantitative PCR

We performed real time PCR analysis to study transcriptional expression levels of the mouse mlc-2a gene. Total RNA was isolated from whole heart tissues of controls ($n = 2$) and Chr 21 chimeric mice ($n = 2$). Synthesized cDNA samples were assayed by real time PCR using TaqMan probes. Representative results confirmed by duplicate analysis are shown in Fig. 2B. No significant differences in mouse *mlc-2a* mRNA expression levels were observed between controls and Chr 21 chimeric mice. These results suggested that *mlc-2a* is post-transcriptionally regulated in heart tissue.

Mouse mlc-2a expression in undifferentiated ES cells

In another experiment, we attempted to study mRNA and protein expression of ES cells containing an extra copy of Chr 21 (ES[Chr 21]) and normal ES cells (control ES), which were used to produce the chimeric mice. However, the mlc-2a protein and its transcript were barely detectable in both ES[Chr 21] and control ES cells (see Figs. 2A and B). This suggested that *mlc-2a* expression could only be observed in differentiated cardiac cells.

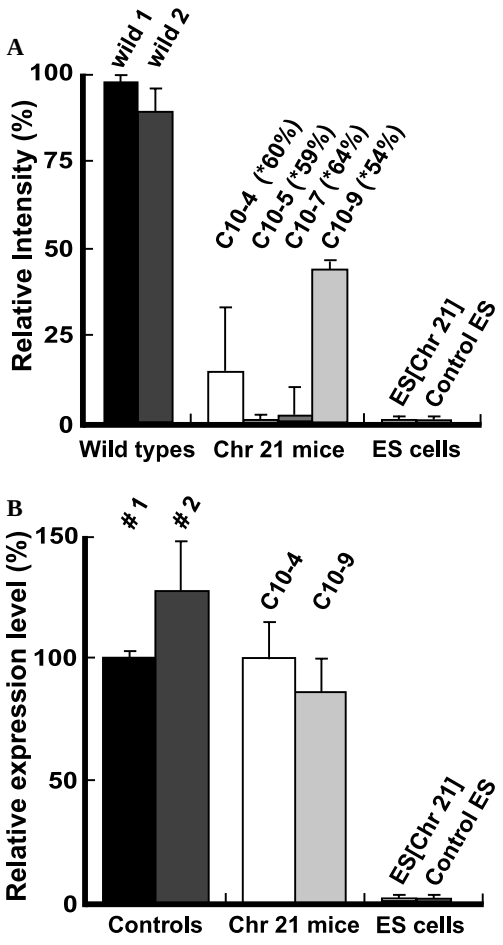

Fig. 2. Quantitative analysis of mlc-2a mRNA and protein expression in Chr 21 chimeric mice. (A) shows expression levels of mouse mlc-2a protein in heart specimens by determination of relative intensity of spots on 2-DE gels. Spots on 2-DE gels in Fig. 1 were detected by silver staining, and analyzed quantitatively by scanning with PDQuest software. Relative intensities were calculated from the density of each spot normalized using the total density of spots. (*) shows retention of Chr 21 in whole heart tissue of chimeric mice. (B) shows the relative expression of mouse mlc-2a in gene transcripts assessed by TaqMan probes. The cDNAs prepared from whole heart tissues of Chr 21 chimeric mice and control mice were analyzed for mlc-2a gene content. The cDNA values normalized to the quantity of cDNA from the control mouse #1 are shown as relative expression level. These experiments were repeated at least three times.

Confirmation of reduced human MLC-2A expression in DS neonates

To confirm quantitatively the level of protein expression of human atrial myosin light chain-2 (MLC-2A), we examined atrium and ventricle samples from DS neonate and normal controls using Western blot analysis. The anti-myosin light chain monoclonal antibody was not specific to MLC-2A, and also cross-reacted with other isoforms of myosin light chain. Thus, proteins obtained from heart tissue were resolved by

2-DE and detected by Western blotting. Consequently, we confirmed the identity of the MLC-2A and ventricular myosin light chain-2 (MLC-2V) protein isoforms by 2-DE and quantitative analysis using the PDQuest 6.2 software. Fig. 3 shows that expression of human MLC-2A from the atrium of DS neonates was suppressed threefold compared to the normal control.

Discussion

In this study, we used Chr 21 chimeric mice derived from ES[Chr 21] cells. These chimeric mice had a wide variety of characteristics similar to DS [7]. We compared protein expression in heart and brain tissues from these mice to those of wild type. We showed that Chr 21 chimeric mice not only expressed the SOD1 protein, derived from an introduced extra copy of Chr 21 in hippocampus, but also showed significant reduction of an endogenous protein, mlc-2a in the heart. The mRNA levels were similar in normal and Chr 21 chimeric mice as analyzed by real time RT-PCR, suggesting that the mlc-2a gene was post-transcriptionally regulated. Moreover, we also confirmed that the expression of the MLC-2A protein was downregulated in human heart atrium of DS neonate.

The size of a single Chr 21 (33.8 Mb) accounts for 1.02% of the total mouse genome size (3300 Mb) [21,22]. However, in this study, the percentage of distinguishable protein spots derived from Chr 21 among total spots on 2-DE gel was 0.12% (1/843) in hippocampus and 0% (0/495) in whole heart. The ratio of differentially expressed proteins in Chr 21 chimeric mouse cells was 10-fold lower than the corresponding genome ratio.

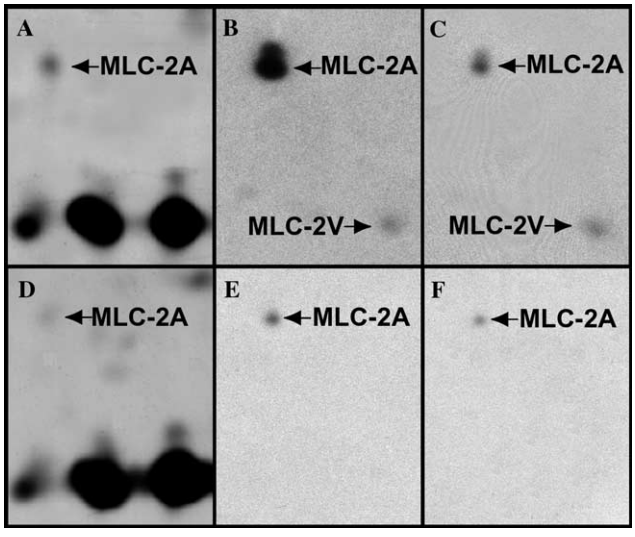

Fig. 3. Protein expression of human MLC-2A in neonatal heart of a DS specimen and normal control. Proteins in neonatal atrium and ventricle of DS and normal controls were separated by 2-DE and detected by silver staining or Western blotting with an anti-myosin light chain (20k) monoclonal antibody. (A)–(C), Atrium samples. (D)–(F), Ventricle samples. (A) and (D), silver-stained normal control. (B) and (E), Western blotting of normal control. (C) and (F), Western blotting of DS specimen. The same volume of protein sample was applied to each gel, (A)–(C) and (D)–(F).

We speculate that in DS model mouse cells, the expression levels of most proteins derived from genes on the extra Chr 21 are unaltered. Instead, expression levels of a gene product derived from a chromosome other than Chr 21 is markedly reduced in certain tissues because of a mass or secondary effect of an extra Chr 21. An extra Chr 21 may change the total balance of gene expression or a few specific genes on Chr 21 may modify gene expression secondarily.

In both humans and mouse, *mlc-2a* is a cardiac-specific gene expressed in the adult atrium and during the earliest stages of cardiac development. In situ hybridization studies during mouse embryogenesis showed that *mlc-2a* was expressed specifically in cardiac tissue throughout days 8–16 postcoitum, with atrial-restricted expression from day 12 and qualitatively greater expression in the atrium than the ventricles from day 9 [23]. The preferential expression of this gene in the atrium occurs prior to chamber septation, and negative-regulation of this gene in the ventricles is likely to be an important step for the maintenance of normal ventricular development [24]. Thus, *mlc-2a* expression corresponds to the activation of the earliest known markers of the cardiac muscle gene program. Consequently, these data suggest that post-transcriptional downregulation of *mlc-2a* in vivo has distinct effects on developmental anomalies in early stages of cardiogenesis, and altered expression of the mlc-2a gene plays a key role in CHD observed in DS.

The mlc-2a gene is located on chromosome 11 in mouse and chromosome 7 in humans, neither of which are implicated in DS (human Chr 21) or DS models (mouse chromosome 16) [18]. To our knowledge, this proteomic-based study is the first demonstration that in cardiac tissue, gene expression on chromosomes other than Chr 21 are affected in mouse models of DS and human DS patients. Previous studies of trisomy 21 have focused on dosage-dependent gene regulation, and showed that expression levels of a gene or gene cluster such as SOD1 and Est-2 increase 1.5-fold in tissue of patients with trisomy 21 [25,26]. However, our data suggest that dosage imbalance due to trisomy 21 has a trans-acting effect and lowers the expression of genes encoded elsewhere in the genome [27].

In hippocampus, we detected differences in expression levels of SOD1 protein, a gene found on Chr 21. *SOD1* is the antioxidant enzyme which catalyzes the dismutation of superoxide to oxygen and hydrogen peroxide, suggesting that the increase of *SOD1* expression in brain is associated with the neurological symptoms observed in DS [26]. However, we did not observe human SOD1

protein expression in cardiac muscle of Chr 21 chimeric mice. Thus, it cannot be concluded that *SOD1* expression induces the DS phenotype in heart. This therefore suggests that *SOD1* does not contribute to the DS phenotype in heart. Furthermore, we could not find any difference in expression levels of Chr 21 proteins between wild type and Chr 21 chimeric mice in the heart tissues. Instead, we noted a difference in protein expression levels of mlc-2a, a gene found on chromosome 11, suggesting the existence of two mechanisms involved in the manifestation of the DS phenotype. The occurrence of these mechanisms was tissue-specific. In hippocampus, genes on Chr 21 might directly affect the phenotype. However, in cardiac muscle, *mlc-2a* possibly contributes more to the DS phenotype as compared to genes found on Chr 21. Other studies using undifferentiated ES cells suggested that differences in mouse *mlc-2a* expression levels could be induced after differentiation of cells into cardiac tissue. Thus, other factors such as state of differentiation are also important in the manifestation of the DS phenotype. We also reported that the introduction of an extra Chr 21 into ES cells may lead to the disturbance of cardiogenesis [13].

We have used human heart tissue to compare protein expression in DS and normal neonate using this proteomic approach. As a result, a variety of candidate spots other than the human mlc-2a protein were detected in DS specimens (data not shown). We suggest that these spots, aside from *mlc-2a*, could represent genetic or phenotypic differences among individuals. In contrast, when we employed a mouse model, only one candidate protein was detected. Thus, protein analysis using Chr 21 chimeric mice is a valid and powerful approach for further understanding the molecular mechanisms underlying the pathogenesis of DS.

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References

[1] C.J. Epstein, Consequences of Chromosome Imbalance: Principles, Mechanisms and Models, Cambridge University Press, New York, 1986.

[2] S. Miyabara, M. Ando, K. Suzumori, M. Nishibatake, N. Saito, H. Sugihara, T. Ikenoue, Incidence and types of congenital cardiovascular malformations in Japanese trisomy 21 fetuses around 20 weeks, Congenital. Anomalies. 40 (2000) 117–122.

[3] J.R. Korenberg, X.N. Chen, R. Schipper, Z. Sun, R. Gonsky, S. Gerwehr, N. Carpenter, C. Daumer, P. Dignan, C. Disteche, et al., Down syndrome phenotypes: the consequences of chromosomal imbalance, Proc. Natl. Acad. Sci. USA 91 (1994) 4997–5001.

[4] K. Yamakawa, Y.K. Huot, M.A. Haendelt, R. Hubert, X.N. Chen, G.E. Lyons, J.R. Korenberg, DSCAM: a novel member of the immunoglobulin superfamily maps in a Down syndrome region and is involved in the development of the nervous system, Hum. Mol. Genet. 7 (1998) 227–237.

[5] G.M. Barlow, X.N. Chen, Z.Y. Shi, G.E. Lyons, D.M. Kurnit, L. Celle, N.B. Spinner, E. Zackai, M.J. Pettenati, J.R. Korenberg, et al., Down syndrome congenital heart disease: a narrowed region and a candidate gene, Genet. Med. 3 (2001) 91–101.

[6] I. Kola, P.J. Hertzog, Animal models in the study of the biological function of genes on human chromosome 21 and their role in the pathophysiology of Down syndrome, Hum. Mol. Genet. 6 (1997) 1713–1727.

[7] T. Shinohara, K. Tomizuka, S. Miyabara, S. Takehara, Y. Kazuki, J. Inoue, M. Katoh, H. Nakane, A. Iino, A. Ohguma, S. Ikegami, K. Inokuchi, I. Ishida, R.H. Reeves, M. Oshimura, Mice containing a human chromosome 21 model behavioral impairment and cardiac anomalies of Down's syndrome, Hum. Mol. Genet. 10 (2001) 1163–1175.

[8] Y. Kuroiwa, K. Tomizuka, T. Shinohara, Y. Kazuki, H. Yoshida, A. Ohguma, T. Yamamoto, S. Tanaka, M. Oshimura, I. Ishida, Manipulation of human minichromosomes to carry greater than megabase-sized chromosome inserts, Nat. Biotechnol. 18 (2000) 1086–1090.

[9] T. Shinohara, K. Tomizuka, S. Takehara, K. Yamauchi, M. Katoh, A. Ohguma, I. Ishida, M. Oshimura, Stability of transferred human chromosome fragments in cultured cells and in mice, Chromosome Res. 8 (2000) 713–725.

[10] Y. Kazuki, T. Shinohara, K. Tomizuka, M. Katoh, A. Ohguma, I. Ishida, M. Oshimura, Germline transmission of a transferred human chromosome 21 fragment in transchromosomal mice, J. Hum. Genet. 46 (2001) 600–603.

[11] R.H. Reeves, N.G. Irving, T.H. Moran, A. Wohn, C. Kitt, S.S. Sisodia, C. Schmidt, R.T. Bronson, M.T. Davisson, A mouse model for Down syndrome exhibits learning and behaviour deficits, Nat. Genet. 11 (1995) 177–184.

[12] H. Sago, E.J. Carlson, D.J. Smith, J. Kilbridge, E.M. Rubin, W.C. Mobley, C.J. Epstein, T.T. Huang, Ts1Cje, a partial trisomy 16 mouse model for Down syndrome, exhibits learning and behavioral abnormalities, Proc. Natl. Acad. Sci. USA 95 (1998) 6256–6261.

[13] T. Inoue, T. Shinohara, S. Takehara, J. Inoue, H. Kamino, H. Kugoh, M. Oshimura, Specific impairment of cardiogenesis in mouse ES cells containing a human chromosome 21, Biochem. Biophys. Res. Commun. 273 (2000) 219–224.

[14] S. Greber, G. Lubec, N. Cairns, M. Fountoulakis, Decreased levels of synaptosomal associated protein 25 in the brain of patients with Down syndrome and Alzheimer's disease, Electrophoresis 20 (1999) 928–934.

[15] T. Toda, N. Kimura, Standardization of protocol for immobiline 2-D PAGE and construction of 2-D PAGE protein database on World Wide Web home page, Jpn. J. Electrophoresis 41 (1997) 13–19.

[16] METHODS in PROTEOMICS including our standard protocol of 2-D PAGE http://proteome.tmig.or.jp/2D/2DE_method.html.

[17] M. Oh-Ishi, T. Hirabayashi, Comparison of protein constituents between atria and ventricles from various vertebrates by two-dimensional gel electrophoresis, Comp. Biochem. Physiol. B 92 (1989) 609–617.

[18] SWISS-PROT (ExPASy Molecular Biology Server) http://www.expasy.org/sprot/.

[19] P. Razeghi, M.E. Young, S. Abbasi, H. Taegtmeyer, Hypoxia in vivo decreases peroxisome proliferator-activated receptor alpha-regulated gene expression in rat heart, Biochem. Biophys. Res. Commun. 287 (1998) 5–10.

[20] M.S. Chaussee, J.M. Musser, et al., Identification of Rgg-regulated exoproteins of Streptococcus pyogenes, Infect. Immun. 69 (2001) 822–831.

[21] M. Hattori, A. Fujiyama, T.D. Taylor, H. Watanabe, T. Yada, H.S. Park, A. Toyoda, K. Ishii, Y. Totoki, D.K. Choi, et al., DNA sequence of human chromosome 21, Nature 405 (2000) 311–319.
[22] T.A. Brown, Molecular Biology Labfax, vol. 1, second ed., Academic Press, London, 1998.
[23] S.W. Kubalak, W.C. Miller-Hance, T.X. O'Brien, E. Dyson, K.R. Chien, Chamber specification of atrial myosin light chain-2 expression precedes septation during murine cardiogenesis, J. Biol. Chem. 269 (1994) 16961–16970.
[24] P.J. Gruber, S.W. Kubalak, K.R. Chien, Downregulation of atrial markers during cardiac chamber morphogenesis is irreversible in murine embryos, Development 125 (1998) 4427–4438.
[25] E. Sanij, T. Hatzistavrou, P. Hertzog, I. Kola, E.J. Wolvetang, Est-2 is induced by oxidative stress and sensitizes cells to H_2O_2-induced apoptosis: implications for Down's syndrome, Biochem. Biophys. Res. Commun. 287 (2001) 1003–1008.
[26] R.C. Iannello, P.J. Crack, J.B. de Haan, I. Kola, Oxidative stress and neural dysfunction in Down syndrome, J. Neural. Transm. Suppl. 57 (1999) 257–267.
[27] J.A. Birchler, U. Bhadra, M.P. Bhadra, D.L. Auger, Dosage-dependent gene regulation in multicellular eukaryotes: implications for dosage compensation, aneuploid syndromes, and quantitative traits, Dev. Biol. 234 (2001) 275–288.