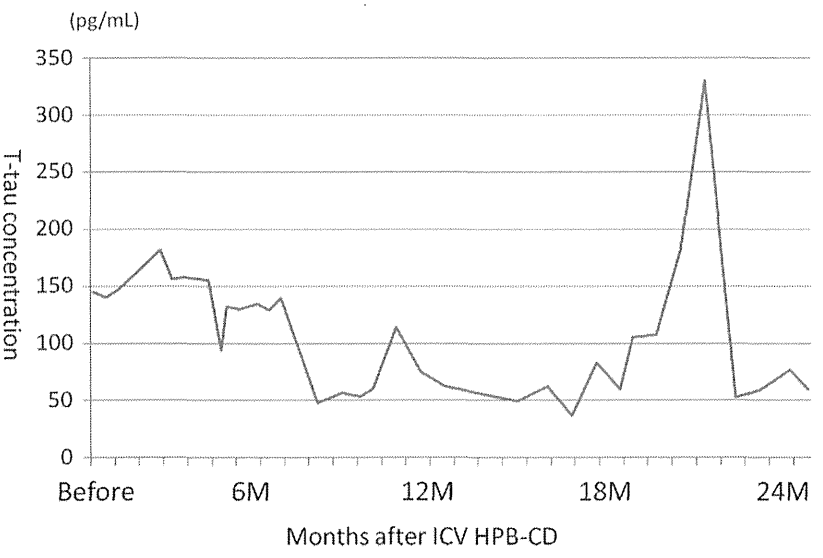


Fig. 5. Changes in CSF T-tau concentration after ICV HPB-CD therapy. The levels of CSF T-tau gradually decreased after the ICV HPB-CD therapy, but were transiently increased from the 21 months to 23 months period, when the dose of HPB-CD reached 300 mg twice a week.

HPB-CD penetrates deeper into the central nervous system beyond the CSF compartment, as suggested by Ottinger et al. in an animal study [8].

The reported ED_{50} in NPC mice is approximately about 0.5 mg/kg, with 35 mg/kg administration of HPB-CD effective for 1 week [4]. An in vitro study using primary cultures of neurons and glial cells from *Npc1*$^{-/-}$ mice also demonstrated that 0.1 mM of HPB-CD is the optimum concentration and that 10 mM is toxic for cells [15]. Our present data combined with these previous observations suggest that 10 mg/kg would be a sufficient dose of ICV HPB-CD, although the optimum intervals for ICV injections of HPB-CD remain unclear. In our experience, the patient looks more alert after several days of HPB-CD injection according to the parents' observation. Thus, we tried twice a week injections of 200 mg HPB-CD. MRS and PET study suggested better results, supported by the observation of slightly lower clearance rates of HPB-CD compared to the CSF turn-over rate of 21–25 mL/h in normal human subjects [13].

In the present study, we showed the efficacy and safety of ICV HPB-CD treatment and the effect on concentrations of HPB-CD in CSF. However, the optimal dose and dosing intervals for ICV HPB-CD remain to be determined, necessitating further studies into the mechanisms of HPB-CD action in NPC.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ymgmr.2014.08.004.

Acknowledgments

The authors thank Dr. Peter Pentchev for introducing them to Chris Hempel, who generously supplied the protocols for the Hempel twins. The authors also thank Dr. Kaori Adachi and Dr. Eiji Nanba, Tottori University, for the genetic diagnosis of the patient. This work was supported by a JSPS KAKENHI Grant Number 23590642.

References

[1] M.T. Vanier, Niemann–Pick disease type C, Orphanet J. Rare Dis. 5 (2010) 16.
[2] B. Liu, S.D. Turley, D.K. Burns, A.M. Miller, J.J. Repa, J.M. Dietschy, Reversal of defective lysosomal transport in NPC disease ameliorates liver dysfunction and neurodegeneration in the npc1 −/− mouse, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 2377–2382.
[3] C.D. Davidson, N.F. Ali, M.C. Micsenyi, G. Stephney, S. Renault, K. Dobrenis, D.S. Ory, M.T. Vanier, S.U. Walkley, Chronic cyclodextrin treatment of murine Niemann–Pick C disease ameliorates neuronal cholesterol and glycosphingolipid storage and disease progression, PLoS One 4 (2009) e6951.
[4] A. Aqul, B. Liu, C.M. Ramirez, A.A. Pieper, S.J. Estill, D.K. Burns, J.J. Repa, S.D. Turley, J.M. Dietschy, Unesterified cholesterol accumulation in late endosomes/lysosomes causes neurodegeneration and is prevented by driving cholesterol export from this compartment, J. Neurosci. 31 (2011) 9404–9413.

[5] B. Liu, Therapeutic potential of cyclodextrins in the treatment of Niemann–Pick type C disease, Clin. Lipidol. 7 (2012) 289–301.
[6] T. Irie, K. Uekama, Pharmaceutical applications of cyclodextrins. III. Toxicological issues and safety evaluation, J. Pharm. Sci. 86 (1997) 147–162.
[7] M. Matsuo, K. Togawa, S. Hirabaru, A. Mochinaga, M. Narita, M. Adachi, T. Egashira, T. Irie, K. Ohno, Effects of cyclodextrin in two patients with Niemann–Pick type C disease, Mol. Genet. Metab. 108 (2013) 76–81.
[8] E.A. Ottinger, M.L. Kao, N. Carrillo-Carrasco, N. Yanjanin, R.K. Shankar, M. Janssen, M. Brewster, I. Scott, X. Xu, J. Cradock, P. Terse, S.J. Dehdashti, J. Marugan, W. Zheng, L. Portilla, A. Hubbs, W.J. Pavan, J. Heiss, C.H. Vite, S.U. Walkley, D.S. Ory, S.A. Silber, F.D. Porter, C.P. Austin, J.C. McKew, Collaborative development of 2-hydroxypropyl-β-cyclodextrin for the treatment of Niemann–Pick type C1 disease, Curr. Top. Med. Chem. 14 (2014) 330–339.
[9] H.W. Frijlink, J. Visser, N.R. Hefting, R. Oosting, D.K. Meijer, C.F. Lerk, The pharmacokinetics of beta-cyclodextrin and hydroxypropyl-beta-cyclodextrin in the rat, Pharm. Res. 7 (1990) 1248–1252.
[10] N. Mattsson, H. Zetterberg, S. Bianconi, N.M. Yanjanin, R. Fu, J.E. Månsson, F.D. Porter, K. Blennow, Gamma-secretase-dependent amyloid-beta is increased in Niemann–Pick type C: a cross-sectional study, Neurology 76 (2011) 366–372.
[11] N. Mattsson, H. Zetterberg, S. Bianconi, N.M. Yanjanin, R. Fu, J.E. Månsson, F.D. Porter, K. Blennow, Miglustat treatment may reduce cerebrospinal fluid levels of the axonal degeneration marker tau in Niemann–Pick type C, JIMD Rep. 3 (2012) 45–52.
[12] S. Ward, P. O'Donnell, S. Fernandez, C.H. Vite, 2-hydroxypropyl-beta-cyclodextrin raises hearing threshold in normal cats and in cats with Niemann–Pick type C disease, Pediatr. Res. 68 (2010) 52–56.
[13] H. Jiang, R. Sidhu, H. Fujiwara, M. DeMeulder, R. de Vries, Y. Gong, M. Kao, F.D. Porter, N.M. Yanjanin, N. Carillo—Carasco, X. Xu, E. Ottinger, M. Woolery, D.S. Ory, X. Jiang, Development and validation of sensitive LC—MS/MS assays for quantification of 2-hydroxypropyl-β-cyclodextrin in human plasma and CSF, J. Lipid Res. 55 (2014) 1537–1548.
[14] D. Maetzel, S. Sarkar, H. Wang, L. Abi-Mosleh, P. Xu, A.W. Cheng, Q. Gao, M. Mitalipova, R. Jaenisch, Genetic and chemical correction of cholesterol accumulation and impaired autophagy in hepatic and neural cells derived from Niemann–Pick type C patient-specific iPS cells, Stem Cell Rep. 2 (2014) 866–880.
[15] K.B. Peake, J.E. Vance, Normalization of cholesterol homeostasis by 2-hydroxypropyl-β-cyclodextrin in neurons and glia from Niemann–Pick C1 (NPC1)-deficient mice, J. Biol. Chem. 287 (2012) 9290–9298.
[16] R. Troncin, C. Dadure, Paediatric spinal anaesthesia, Updat. Anaesth. 25 (2009) 22–24.

STEM CELLS®

EMBRYONIC STEM CELLS/INDUCED PLURIPOTENT STEM CELLS

Isogenic Induced Pluripotent Stem Cell Lines from an Adult with Mosaic Down Syndrome Model Accelerated neuronal Ageing and Neurodegeneration

Aoife Murray[1,2], Audrey Letourneau[3], Claudia Canzonetta[1], Elisavet Stathaki[4], Stefania Gimelli[4], Frederique Sloan-Bena[4], Robert Abrehart[1], Pollyanna Goh[1,2], Shuhui Lim[5], Chiara Baldo[6], Franca Dagna-Bricarelli[7†], Saad Hannan[8], Martin Mortensen[8], David Ballard[9], Denise Synder-combe Court[9], Noemi Fusaki[10#], Mamoru Hasegawa[11], Trevor G. Smart[8], Cleo Bishop[1], Stylianos E. Antonarakis[3], Jürgen Groet[1,2,12*], and Dean Nizetic[1,2,5*]

[1]The Blizard Institute, Barts and The London School of Medicine, Queen Mary University of London, UK; [2]The LonDownS Consortium, Wellcome Trust, UK; [3]Department of Genetic Medicine and Development, University of Geneva Medical School, Geneva, Switzerland; [4]Service of Genetic Medicine, University Geneva Hospitals, Geneva, Switzerland; [5]Lee Kong Chian School of Medicine, Nanyang Technological University, Singapore; [6]Human Genetics Laboratory, Galliera Hospital, Genoa, Italy; [7]Liguria Department of Genetics, Genoa, Italy; [8]Department of Neuroscience, Physiology and Pharmacology, University College London, UK; [9]Department of Forensic and Analytical Science, King's College, London, UK; [10]Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, Saitama, Japan; [11]DNAVEC corporation, Ibaraki, Tokyo, Japan; [12] Stem Cell Laboratory, National Centre for Bowel Research and Surgical Innovation, Queen Mary University of London, UK
Correspondence to: Professor Dean Nizetic M.D. Ph.D., Lee Kong Chian School of Medicine, Nanyang Technological University Singapore, Proteos Building, Level 4, 61 Biopolis Drive, Biopolis, Singapore 138673, E-mail: d.nizetic@ntu.edu.sg, Tel: +65 6778 4728, Fax: +65 6778 5824 And Dr Jurgen Groet, PhD, The Blizard Institute, Barts & The London School of Medicine, Blizard Building, 4 Newark Street, London E1 2AT, United Kingdom, E-mail: j.groet@qmul.ac.uk, Tel: +442078822271, Fax: +442078822183; [†]sadly deceased during revision of this manuscript; [#]Present address: Dept. of Opthalmology, Keio University School of Medicine, Tokyo, Japan; *co-corresponding authors; Grants supporting this work: Kay Kendall Leukaemia Fund, Fondation Jerome, Lejeune, The Wellcome Trust "LonDownS Consortium" Strategic Funding, Award to DN, the "AnEUploidy Consortium" grant from Framework Programme, 6 from the EU Commission to SEA and DN, SNF and ERC grants to SEA, Telethon, Network of Genetic Biobanks (Telethon project GTB12001)

Received October 12, 2014; accepted for publication January 17, 2015; available online without subscription through the open access option.

1066-5099/2015/$30.00/0
This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/stem.1968

Key words. Down syndrome • neurodegeneration • neurogenesis • neuronal differentiation

ABSTRACT

Trisomy 21 (T21), Down Syndrome (DS) is the most common genetic cause of dementia and intellectual disability. Modelling DS is beginning to yield pharmaceutical therapeutic interventions for amelioration of intellectual disability, which are currently being tested in clinical trials. DS is also a unique genetic system for investigation of pathological and protective mechanisms for accelerated ageing, neurodegeneration, dementia, cancer and other important common diseases. New drugs could be identified and disease mechanisms better understood by establishment of well controlled cell model systems. We have developed a first non-integration-reprogrammed isogenic human induced-pluripotent-stem-cells (iPSC) model of DS by reprogramming the skin fibroblasts from an adult individual with constitutional mosaicism for DS, and separately cloned multiple isogenic T21 and euploid (D21) iPSC lines. Our model shows a very low number of reprogramming rearrangements as assessed by a high-resolution whole genome CGH-array hybridisation and it reproduces several cellular pathologies seen in primary human DS cells, as assessed by automated high-content microscopic analysis. Early differentiation shows an imbalance of the lineage-specific stem/progenitor cell compartments: T21 causes slower proliferation of neural and faster expansion of hematopoietic lineage. T21 iPSC-derived neurons show increased production of amyloid peptide-containing material, a decrease in mitochondrial membrane potential, and an increased number and abnormal appearance of mitochondria. Finally, T21-derived neurons show significantly higher number of DNA double-strand breaks than isogenic D21 controls. Our fully isogenic system therefore opens possibilities for modelling mechanisms of developmental, accelerated ageing, and neurodegenerative pathologies caused by T21.
STEM CELLS *2014; 00:000–000*

Introduction

Trisomy 21 (T21), Down Syndrome (DS) is the most common genetic cause of intellectual disability and dementia with rising global prevalence [1]. Several phenotypes have been observed at molecular and cellular level, that reproduce in primary tissues from human individuals with T21. Some of these cellular phenotypes directly map onto clinical components of DS; these include intellectual disability, defects in cognitive development and age-related cognitive decline, Alzheimer's disease (AD)-like dementia, epilepsy, congenital heart defect, childhood leukemia and others [2-12]. Modelling DS is beginning to yield pharmaceutical therapeutic interventions for amelioration of intellectual disability which are currently being tested in clinical trials. New drugs could be identified by high throughput screening of chemical libraries using cellular assays, and therefore well controlled cellular model systems are required. In order to eliminate effects of wide phenotypic differences among individuals with DS, the requirement for many experimental purposes has become the use of an isogenic iPSC model for DS, where the sole difference between iPSC lines is the presence of the third chromosome 21.

Several recent iPSC models of DS have been developed. All of these (with one exception) used integrational reprogramming. All of these reprogrammed cells were derived from fetal, neonatal, or 1 year old infant DS, actually, three studies [13-15] used the same iPSC lines as a starting point [16]. Some of these studies report defects in NPC proliferation [17], neurogenesis [13], gliogenesis and neurite outgrowth [18], others defects in synaptic morphology and function, mitochondrial dysfunction [19] and increased amyloid deposition [15]. Other models show increased propensity to generate hematopoietic precursors and increased multilineage myeloid hematopoiesis potential [14,20]. Most of these results have been generated on non-isogenic comparisons, with isogenic lines either serendipitously generated in cell culture [14,19], or using an ingenious but complex and laborious approach to silence the third chromosome 21 [13,21]. In some cases, non-isogenic lines were pooled with one isogenic line [14,19]. In one study, a unique case of heterokaryotypic twin fetal cells was exploited [17]. None of these papers reported a genome-wide high-resolution array-Comparative Genomic Hybridization (aCGH) analysis of the resulting iPSCs for the rigorous measurement of the artificial copy-number rearrangements known to frequently occur during the reprogramming process.

Here we present the first iPSC DS model which is both non-integrationally reprogrammed and fully isogenic, and the first derived from an adult individual with DS which is a constitutional mosaic. Furthermore, we verify the high level of genome integrity of the resulting iPSCs, by showing a very low number of reprogramming rearrangements as assessed by a high-resolution whole genome aCGH, and we make entirely isogenic comparisons of 3 trisomic and 3 disomic iPSC lines derived from this model. This approach minimizes the influence of any copy number fluctuations additional to T21, and eliminates genotypic difference noise, allowing the "clean" detection of T21-causing effects. Our model reproduces several T21 cellular pathologies seen in primary human DS cells, but hitherto not reported in iPSC models, such as abnormalities in mitochondrial number and size, and an increase in DNA double-strand breaks in neurons.

Materials and Methods

Detailed methods are shown in Supplementary Data online.

Results

We generated the iPSCs by non-integration reprogramming using temperature-sensitive Sendai virus [22], from primary skin fibroblasts from a young adult diagnosed with constitutional mosaicism for DS (strategy illustrated in Supplementary Data Fig. S1). We isolated individual clones (labelled as C[number]), expanded them, and confirmed that they tested positive for Alkaline-phosphatase expression, and the presence of markers of pluripotency (Fig. 1A). The Sendai virus was efficiently removed after 7-10 passages (Fig. 1B). De-methylation of the endogenous *NANOG* promoter in the iPSCs, compared to the parental skin fibroblasts, was established via bisulfite sequencing (Fig. 1C). Individual clones were analyzed by rigorous whole-genome microsatellite DNA fingerprinting, which established the presence of clones with T21, and euploid genome (D21), which are otherwise isogenic (Fig. 1D). In a preliminary RNA-seq experiment, the isogenic iPSCs show an expected increase in transcript levels for the majority of HSA21 genes (not shown). The genome integrity of the resulting iPSCs is of a high level, as was assessed by high-resolution, whole genome aCGH (Supplementary Data Fig. S2). The supernumerary HSA21 is intact and complete in both analyzed trisomic lines (T21C5 and T21C6), and T21 is stable for at least 17-19 passages, which is as far as we tested for the presence of T21 (Supplementary Data Fig. S2A). After filtering out the copy number variations (CNVs) that occur commonly in healthy individuals (using comparison to the Database of Genomic Variation, DGV) we made an *in silico* comparison with the published survey of genome-rearrangement artefacts in iPSC generated by conventional integration-reprogramming [23]. Selecting only events at the same aCGH resolution and the same passage number as in our study, we detected a significantly lower number of un-common CNVs, affecting a significantly lower number of genes in our lines, relative to those generated by classical integrational reprogram-

ming methods (Supplementary Data Fig. S2B and S2C). In compliance with international guidelines for iPSC nomenclature [24] we name these iPSCs: NIZEDSM1iD21-C3, -C7 and -C9 for the disomic lines, respectively, and NIZEDSM1iT21-C5, -C6, -C13 for the trisomic lines, respectively (henceforward abbreviated to D21C3, D21C7, D21C9, T21C5, T21C6 and T21C13). Microsatellite DNA fingerprinting was repeated at later passages and confirmed that trisomy 21 is retained through routine passaging (Supplementary Data Fig. S2D). The isogenic DS iPSC clones can differentiate into cell lineages of all 3 embryonic layers both *in vitro* and *in vivo* (Supplementary Data Fig. S3).

After 45 days in culture, using neuronal differentiation via Neuro-EB protocol (see Supplementary Methods), both disomic and trisomic neuronal differentiation cultures were able to produce mature looking neurons expressing βIII-tubulin, the inhibitory neurotransmitter GABA, as well as pre- and post-synaptic markers of excitatory synapses, PSD95 and VGlut (Supplementary Data Fig. S4A, S4B, respectively). In order to accelerate neuronal differentiation and improve yields, we applied a directed neuronal differentiation protocol using dual SMAD inhibition (Noggin and SB431542), combined with stimulation of retinoid signaling and the addition of the Sonic Hedgehog agonist purmorphamine [25]. Both trisomic and disomic lines were able to produce electrophysiologically-active neurons (Supplementary Data Fig. S4C) that supported spontaneous action potential firing and functional whole-cell current responses to saturating concentrations of externally-applied GABA and glycine, observed in neurons from both D21C3 (at 28 days) and T21C5 lines (at 40 days). In order to gain an approximate estimate of the proportion of neurons likely to fire spontaneous action potentials, we used Ca2+ imaging and observed multiple Ca2+ transients, which would be indicative of regenerative spontaneous activity in accord with the firing of spontaneous action potentials (FigS5A,B). Quantification of the numbers of neurons exhibiting calcium transients over the 1 minute time-course showed no significant difference between D21 and T21 neurons (Fig S5B). In addition, under voltage-clamp conditions, we identified neuronal sensitivities to GABA and glycine which indicates the cell surface expression of functional GABAA and glycine receptors. This was apparent in most cells tested, with no difference noted between T21 and D21 cells (not shown).

To examine early stages of iPSC differentiation an embryoid body (EB) protocol was adopted for both hematopoietic and neuronal differentiation, modifying the published method [26]. For hematopoietic EBs (HEBs) 3,000 live cells in single cell suspension were allowed to aggregate in a 96-well. Imaging and analysis of HEBs after 5 days showed that T21 HEBs were significantly larger than the euploid controls (Supplementary Data Fig. S6A). This increase in HEB size was caused by an increase in the cell numbers (Fig. S6B), which at this stage are predicted to be early haematopoietic mesoderm precursors. A similar result was obtained with early T21 mesodermal colonies derived from transchromosomic mouse ES cells [7]. Although the T21 HEBs gave a higher proportion of CD34+ cells (haematopoietic stem/progenitor lineages) on both days 12 and 18 of further differentiation (not shown), there was no statistically significant difference. By contrast, neural progenitor cells (NPCs) derived from neuro EBs (NEBs) showed a reduction in proliferation rate. The cumulative population doublings of trisomic cells was significantly reduced during the expansion of NPCs (Supplementary Data Fig. S6C). Increased cell death is unlikely to be the cause of the observed decrease population cell doubling, because the proportion of non-viable cells in the same counts shows no increase in T21 NPCs (Fig S6D).

iPSC-derived neurons were analysed after 60 days of differentiation following the dual SMAD inhibition protocol (mentioned previously, but here minus purmorphamine) that generates multiple classes of projection neurons from different cortical layers [25]. We observed the typical morphology of neurogenic cortical rosettes as published [27], and confirmed that after 60 days neuronal differentiation, >95% of cells were Tuj1+ (not shown). As this protocol generated 100% excitatory glutamatergic neurons [25,27], and as no differences in the composition of cortical layer sub-populations were observed between DS and normal hiPSCs [15], we think it unlikely that difference in cell type profiles would be responsible for the differences between T21 and D21 observed in our results, though we cannot fully rule out this possibility. Immunostaining of fixed neurons with an antibody (6E10) raised against the epitopes in the Aβ–peptide, but reactive to all proteolytic fragments of the amyloid precursor protein (APP) that contain this epitope, revealed an increase in total amyloid staining in trisomic neurons compared to isogenic euploid controls (Fig. 2A and B). This demonstrates that increased amyloid production can be successfully modelled in these cells. Trisomic neurons also appear to show an increase in size and number of 6E10-reactive discreet punctiform aggregates (not quantified, Fig. 2B zoom). Our staining does not permit us to conclude if these aggregates are intracellular or extracellular.

To assess mitochondrial membrane potential in neurons, live cells were labelled with JC-10 (Fig. 3A). In healthy cells JC-10 selective accumulates in the mitochondria and forms aggregates with a characteristic fluorescent emission at 590 nm (orange/red). If mitochondrial membrane potential is decreased (due to damaged/unhealthy cells) JC-10 monomers are formed, which are released into the cytoplasm, resulting in a shift to green emission at 525 nm. An increase in both the size and number of mitochondria were observed in the trisomic neurons (Fig. 3B and C), consistent with a previous study in primary T21 neurons which showed "generalized perturbations" in T21 mitochondrial structure and function, including a more fragmented mitochondrial network [9]. Trisomic neurons also showed a

decreased mitochondrial membrane potential, as evidenced by the increase in green cytoplasmic staining with JC-10 (Fig. 3D).

Having shown that T21 neurons have a deficit in mitochondrial function similar to primary human T21 neurons *in vitro*, we anticipate that reactive oxygen species (ROS) production is increased in T21 neurons. Increased ROS leads to increased DNA damage, as measured by the proportion of γH2AX foci, an *in vitro* marker of ageing, shown increased in primary fibroblasts from old, compared to young humans [28], and in primary neurons from old, compared to young mice [29]. We assessed the number of γH2AX foci (Fig. 4A). Fully automated quantification of approximately 18,000 cells per cell line showed a significantly increased number of γH2AX puncta per cell in T21 neurons (Fig. 4B).

Discussion

In conclusion, we report a first non-integration-reprogrammed isogenic and high genomic fidelity iPSC model from an adult with mosaic DS. The model reproduces several differentiation, ageing and neurodegeneration-related cellular phenotypes associated with DS pathology, and attributable solely to T21 as a cause.

Particularly intriguing are two observations (Fig. 3 and Fig. 4) that open a whole set of new interesting mechanistic questions. Increased number of mitochondria in neurons could be related to increased mitochondrial fragmentation observed in primary DS cortical neurons in culture [9]. On the other hand, it is possible that T21 mitochondria in DS neurons, which are high consumers of energy exclusively derived from oxidative metabolism, are hypofunctional (as has been observed in primary DS cortical neurons in culture [9]), and the demand for more energy increases mitochondrial biogenesis. These hypotheses remain to be tested. Increased number of DNA double-strand breaks in T21 neurons (Fig. 4) could be further exploited as a cellular marker of accelerated ageing observed in DS [10,30]. Alternatively, more genomic instability in the nuclei of post-mitotic T21 neurons (such as transposition events [31]) could also explain this observation.

Currently, several interdisciplinary consortia have been organized to study DS genetics and cellular models integrated with the assessment of the adult population with DS for neurocognitive function, age-related decline, presence or absence of dementia and other co-morbidities (for example, see http://www.ucl.ac.uk/london-down-syndrome-consortium, http://www.psychiatry.cam.ac.uk/ciddrg/research/dementia-in-downs-syndrome-dids/). It is therefore important to verify that cellular phenotypes under-pinning DS pathology can also be reproduced in iPSCs derived from an adult individual with DS, as this had not been reported so far.

Up to 75% of constitutionally T21 concepti spontaneously die in utero [10,32]suggesting that in random allelic variation, the presence of a third copy of HSA21 has a 75% probability to cause severe phenotypes that are normally missed. In constitutional mosaicism, T21 with an otherwise deleterious genotype could be rescued by a significant (>50%) presence of normal (D21) cells, which results in varying intensity of clinical DS defects, often not correlating with the percentage of trisomic cells in tissues. This presents with a theoretical (but so far never explored) rationale for T21/D21 cells derived from a constitutionally mosaic DS individual to show more contrasting differences in cellular phenotypes, than comparisons between cells of liveborn 100% trisomic individuals. Such phenotypes are more likely to be robust, reproducible, and therefore more amenable to developing into high throughput screening assays.

Acknowledgments

This work was supported by the project grants from the Kay Kendall Leukaemia Fund (KKL632), and Fondation Jerome Lejeune (2011B/960), by The Wellcome Trust "LonDownS Consortium" Strategic Funding Award (098330/Z/12/Z), and the Lee Kong Chian School of Medicine, Nanyang Technological University-Singapore start-up funding grant M4230024 to DN, by the "AnEUploidy Consorium" grant from Framework Programme 6 from the EU Commission to DN and SEA, as well as SNF 144082 and ERC 249968 grants to SEA. We also thank Barts and The London Charity and the grant 520/1489: "Development of a National Centre for Bowel Research and Surgical Innovation (NCBRSI)" for purchase of high content imaging equipment. Galliera Genetic Bank as member of the Telethon Network of Genetic Biobanks (Telethon project GTB12001) provided us with specimens. We thank Li Pin for help in setting up the iPSC lab at LKCMedicine in Singapore. We thank Gary Warnes for help with the FACS analysis, and Dominique Bonnet, Silvia Marino and the BSU Whitechapel for help in using NOD/SCID mice. We dedicate this paper to Prof. Franca Dagna-Bricarelli, our colleague and co-author who sadly passed away during revision of this manuscript. Her dedicated work in human genetics made all this possible and will remain inspiration to all of us.

AUTHOR CONTRIBUTIONS

A.M.: Conception and design, Collection and assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; A.L.: Conception and design, Collection and assembly of data, Data analysis and interpretation, Manuscript writing; C.C.: Conception and design, Collection and assembly of data, Data analysis and interpretation; E.S.: Collection of data; S.G.: Collection of data; F.S.-B.: Collection of data, Data analysis and interpretation; R.A.: Collection of data; P.G.: Collection of data, Data analysis and interpretation; S.L.: Data analysis and interpretation; C.B.: Provision of study material or patients; F.D.-B.: Provision of study material or patients; S.H.: Collection of data, Data analysis and interpretation; M.M.: Collection of data, Data analysis and interpretation; D.B.: Collection of data, Data analysis and interpretation; D.S.-C.: Collection of data, Data analysis and interpretation; N.F.: Provision of study material; M.H.: Provision of study material; T.S.: Assembly of data, Data analysis and interpretation; C.B.: Collection and assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; S.A.: Financial support, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; J.G.: Conception and design, Collection and assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript; D.N.: Conception and design, Financial support, Collection and assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare no conflict of interest

REFERENCES

1 Epstein CJ. 2001 William Allan Award Address. From Down syndrome to the "human" in "human genetics". *American journal of human genetics.* Feb 2002;70(2):300-313.

2 Antonarakis SE, Lyle R, Dermitzakis ET, Reymond A, Deutsch S. Chromosome 21 and down syndrome: from genomics to pathophysiology. *Nat Rev Genet.* Oct 2004;5(10):725-738.

3 Bahn S, Mimmack M, Ryan M, et al. Neuronal target genes of the neuron-restrictive silencer factor in neurospheres derived from fetuses with Down's syndrome: a gene expression study. *Lancet.* Jan 26 2002;359(9303):310-315.

4 Busciglio J, Yankner BA. Apoptosis and increased generation of reactive oxygen species in Down's syndrome neurons in vitro. *Nature.* Dec 21-28 1995;378(6559):776-779.

5 Canzonetta C, Mulligan C, Deutsch S, et al. DYRK1A-dosage imbalance perturbs NRSF/REST levels, deregulating pluripotency and embryonic stem cell fate in Down syndrome. *American journal of human genetics.* Sep 2008;83(3):388-400.

6 Cataldo AM, Mathews PM, Boiteau AB, et al. Down syndrome fibroblast model of Alzheimer-related endosome pathology: accelerated endocytosis promotes late endocytic defects. *Am J Pathol.* Aug 2008;173(2):370-384.

7 De Vita S, Canzonetta C, Mulligan C, et al. Trisomic dose of several chromosome 21 genes perturbs haematopoietic stem and progenitor cell differentiation in Down's syndrome. *Oncogene.* Nov 18 2010;29(46):6102-6114.

8 Delom F, Burt E, Hoischen A, et al. Transchromosomic cell model of Down syndrome shows aberrant migration, adhesion and proteome response to extracellular matrix. *Proteome Sci.* 2009;7:31.

9 Helguera P, Seiglie J, Rodriguez J, Hanna M, Helguera G, Busciglio J. Adaptive downregulation of mitochondrial function in down syndrome. *Cell Metab.* Jan 8 2013;17(1):132-140.

10 Nizetic D, Groet J. Tumorigenesis in Down's syndrome: big lessons from a small chromosome. *Nature reviews. Cancer.* Oct 2012;12(10):721-732.

11 Roper RJ, Baxter LL, Saran NG, Klinedinst DK, Beachy PA, Reeves RH. Defective cerebellar response to mitogenic Hedgehog signaling in Down's syndrome mice. *Proc Natl Acad Sci U S A.* Jan 31 2006;103(5):1452-1456.

12 Wang Y, Mulligan C, Denyer G, et al. Quantitative proteomics characterization of a mouse embryonic stem cell model of Down syndrome. *Molecular & cellular proteomics : MCP.* Apr 2009;8(4):585-595.

13 Jiang J, Jing Y, Cost GJ, et al. Translating dosage compensation to trisomy 21. *Nature.* Aug 15 2013;500(7462):296-300.

14 Maclean GA, Menne TF, Guo G, et al. Altered hematopoiesis in trisomy 21 as revealed through in vitro differentiation of isogenic human pluripotent cells. *Proc Natl Acad Sci U S A.* Oct 23 2012;109(43):17567-17572.

15 Shi Y, Kirwan P, Smith J, MacLean G, Orkin SH, Livesey FJ. A human stem cell model of early Alzheimer's disease pathology in Down syndrome. *Sci Transl Med.* Mar 7 2012;4(124):124ra129.

16 Park IH, Arora N, Huo H, et al. Disease-specific induced pluripotent stem cells. *Cell.* Sep 5 2008;134(5):877-886.

17 Hibaoui Y, Grad I, Letourneau A, et al. Modelling and rescuing neurodevelopmental defect of Down syndrome using induced pluripotent stem cells from monozygotic twins discordant for trisomy 21. *EMBO molecular medicine.* Feb 2014;6(2):259-277.

18 Briggs JA, Sun J, Shepherd J, et al. Integration-free induced pluripotent stem cells model genetic and neural developmental features of down syndrome etiology. *Stem Cells.* Mar 2013;31(3):467-478.

19 Weick JP, Held DL, Bonadurer GF, 3rd, et al. Deficits in human trisomy 21 iPSCs and neurons. *Proc Natl Acad Sci U S A.* Jun 11 2013;110(24):9962-9967.

20 Chou ST, Byrska-Bishop M, Tober JM, et al. Trisomy 21-associated defects in human primitive hematopoiesis revealed through induced pluripotent stem cells. *Proc Natl Acad Sci U S A.* Oct 23 2012;109(43):17573-17578.

21 Li LB, Chang KH, Wang PR, Hirata RK, Papayannopoulou T, Russell DW. Trisomy correction in down syndrome induced pluripotent stem cells. *Cell Stem Cell.* Nov 2 2012;11(5):615-619.

22 Ban H, Nishishita N, Fusaki N, et al. Efficient generation of transgene-free human induced pluripotent stem cells (iPSCs) by temperature-sensitive Sendai virus vectors. *Proc Natl Acad Sci U S A.* Aug 23 2011;108(34):14234-14239.

23 Hussein SM, Batada NN, Vuoristo S, et al. Copy number variation and selection during reprogramming to pluripotency. *Nature.* Mar 3 2011;471(7336):58-62.

24 Luong MX, Auerbach J, Crook JM, et al. A call for standardized naming and reporting of human ESC and iPSC lines. *Cell Stem Cell.* Apr 8 2011;8(4):357-359.

25 Shi Y, Kirwan P, Livesey FJ. Directed differentiation of human pluripotent stem cells to cerebral cortex neurons and neural networks. *Nat Protoc.* Oct 2012;7(10):1836-1846.

26 Ng ES, Davis RP, Hatzistavrou T, Stanley EG, Elefanty AG. Directed differentiation of human embryonic stem cells as spin embryoid bodies and a description of the hematopoietic blast colony forming assay. *Current protocols in stem cell biology.* Jan 2008;Chapter 1:Unit 1D 3.

27 Shi Y, Kirwan P, Smith J, Robinson HP, Livesey FJ. Human cerebral cortex development from pluripotent stem cells

to functional excitatory synapses. *Nat Neurosci.* Mar 2012;15(3):477-486, S471.

28 Miller JD, Ganat YM, Kishinevsky S, et al. Human iPSC-based modeling of late-onset disease via progerin-induced aging. *Cell Stem Cell.* Dec 5 2013;13(6):691-705.

29 Jurk D, Wang C, Miwa S, et al. Postmitotic neurons develop a p21-dependent senescence-like phenotype driven by a DNA damage response. *Aging cell.* Dec 2012;11(6):996-1004.

30 Adorno M, Sikandar S, Mitra SS, et al. Usp16 contributes to somatic stem-cell defects in Down's syndrome. *Nature.* Sep 19 2013;501(7467):380-384.

31 Erwin JA, Marchetto MC, Gage FH. Mobile DNA elements in the generation of diversity and complexity in the brain. *Nature reviews. Neuroscience.* Aug 2014;15(8):497-506.

32 Spencer K. What is the true fetal loss rate in pregnancies affected by trisomy 21 and how does this influence whether first trimester detection rates are superior to those in the second trimester? *Prenat Diagn.* Sep 2001;21(9):788-789.

See www.StemCells.com for supporting information available online. STEM CELLS; *00:000–000*

Figure 1. Isogenic iPSC model of Down Syndrome generated by reprogramming primary human skin fibroblasts from an adult individual with mosaic Down Syndrome, using a temperature-sensitive Sendai virus.
A: Images of undifferentiated iPSC colonies from two clones (D21C3, T21C5) after 3 passages; brightfield microphotographs and Alkaline Phosphatase expression. Further images after immunohistochemistry for pluripotency markers (SSEA4, TRA 1-60, TRA 1-81). B: Spontaneous elimination of the temperature sensitive, non-integrating Sendai virus (Ts-SeV) from iPSC cells through routine passaging. Primary human skin fibroblasts were infected with the Ts-SeV for positive control, and were stained with an antibody against the SeV protein HN-IL4.1 alongside the iPSC colonies at the indicated passage numbers (P[n]). The agarose gel shows amplification products after RT-PCR, using SeV (and GAPDH) specific primers 1) D21C3 P5, 2) T21C5 P7, 3) T21C5 P10, 4) D21C3 P10, 5) untransfected fibroblasts, 6) SeV infected fibroblasts P0, 7) SeV infected fibroblasts P0 – reverse transcriptase, 8) H_2O C: Demethylation of endogenous *NANOG* promoter following reprogramming: bisulfite sequencing analysis of eight CpG dinucleotides in the promoter region of *NANOG* using genomic DNA isolated from iPSC D21C3 and T21C5, compared to genomic DNA isolated from the primary mosaic DS skin fibroblasts that were used for reprogramming. D: (Left hand panels): Graphs showing semi-quantitative microsatellite PCR analysis for two chromosome 21 (HSA21) markers and for markers from two euploid chromosomes (HSA5, 18) using genomic DNA isolated from iPSC clones. Clones T21C5 and T21C6 are trisomic and Clone D21C3 disomic for HSA21. (Right hand panels): Whole genome microsatellite fingerprint of genomic DNA isolated from iPSC clones, demonstrating that they are isogenic.

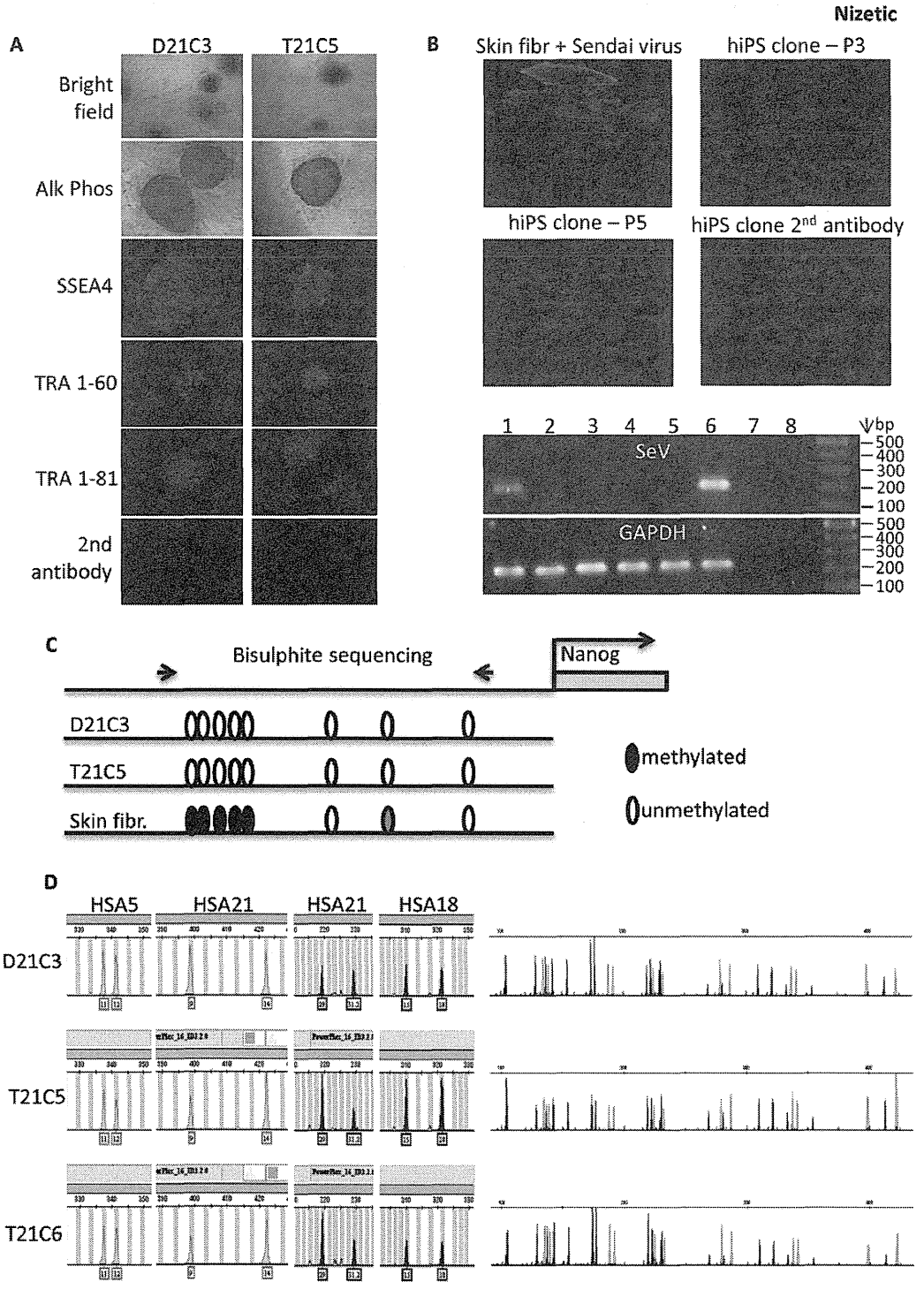
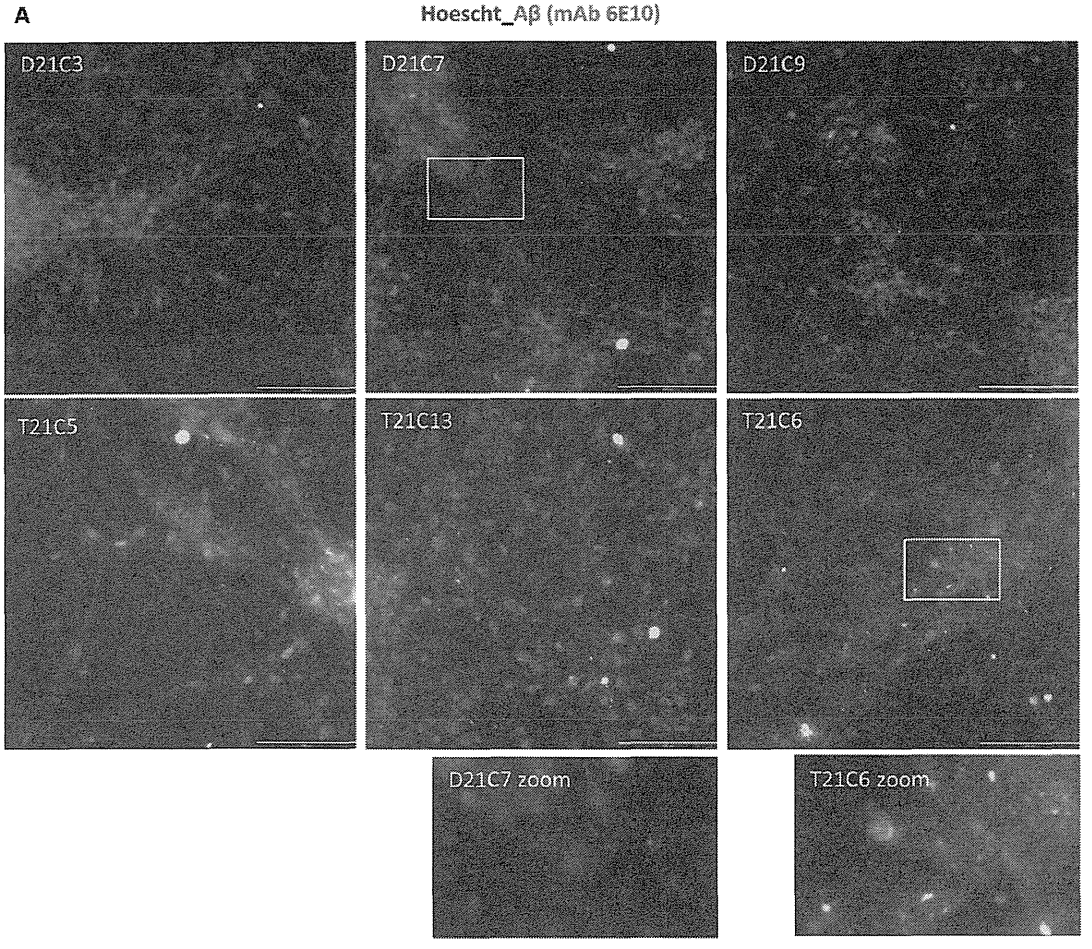


Fig. 1

Figure 2. Trisomy 21 causes an increase in β-amyloid containing material in and around neurons generated from iPSCs.

A: Neurons were generated from iPSCs over a 60 day differentiation protocol. Cells were then fixed and stained with an anti-amyloid peptide antibody (6E10), which is reactive to amino acids 1-16 in β-amyloid, but detects all APP polypeptide forms that contain the epitope. Nuclei were labelled with Hoechst. Scale bar: 100 μm. B: Quantification of the integrated intensity for the 6E10 stain shows an increase of APP expression in T21 neurons compared to the isogenic D21 neurons. Image capture and quantification was performed using automated multi-parametric analysis on the ImageXpress Micro XL (Molecular Devices) wide-field high content imaging system, and data analyzed using MetaMorph software. Three wells per cell line and a minimum of 6,000 cells per well were analyzed. Student's T-test, error bars SEM. Visually, T21 neurons appear to also show more 6E10-reactive aggregates (not quantitated). Zoomed-in images for T21C6 and D21C7 are shown at the same size and magnification.

Nizetic



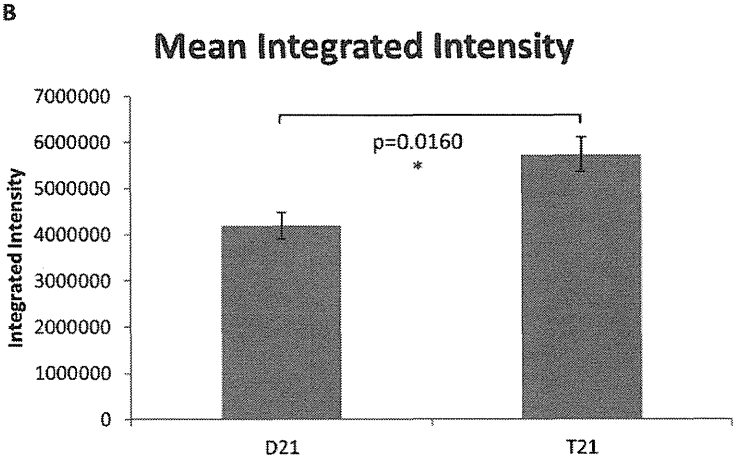


Fig. 2

Figure 3. Trisomy 21 results in an increase in size and number of mitochondria in neurons generated from iPSCs.
A: Neurons were generated from iPSCs over a 60 day differentiation protocol. Live cells were then loaded with JC-10 to assess mitochondrial membrane potential. Healthy mitochondria are labelled in red, while green cytoplasmic staining indicates that JC-10 is diffusing out of the mitochondria due to decreased mitochondrial membrane potential. Representative images for each cell line are shown. Image capture and quantification was performed using automated multi-parametric analysis on the ImageXpress Micro XL (Molecular Devices) wide-field high content imaging system, and data analyzed using MetaMorph software. A total of 4 wells and a minimum of 1,500 cells per cell line were imaged and analyzed. Scale bar: 100 μm (identical scale for all images). B: Quantification of the number of mitochondria per cell, and (C) the mean mitochondrial area show that both are increased in T21 neurons compared to the isogenic D21 neurons. D: Quantification of the integrated intensity for the green signal generated by JC-10 shows decreased mitochondrial membrane potential in T21 neurons compared to the isogenic D21 neurons. Student's T-test, error bars SEM.

Nizetic

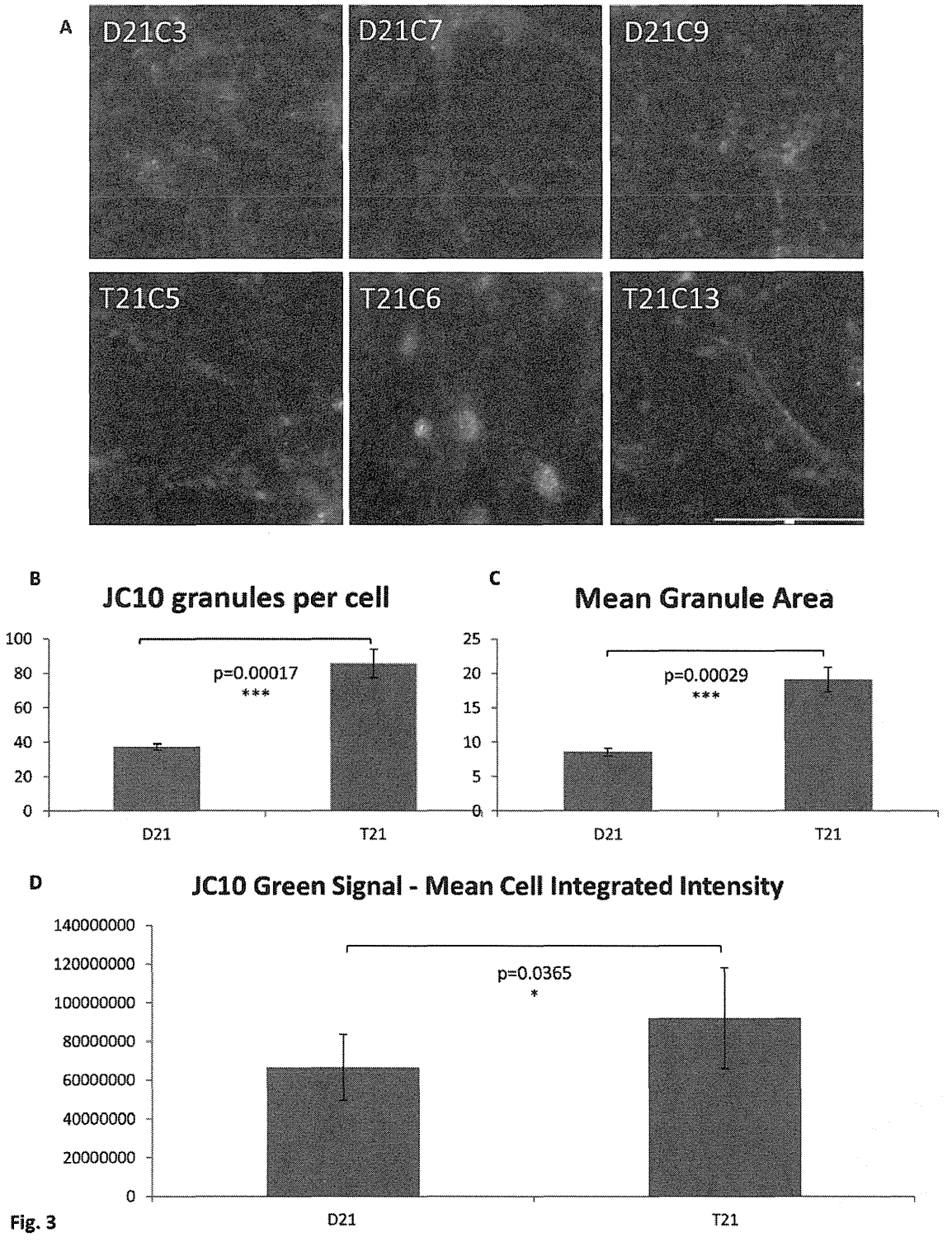


Fig. 3

Figure 4. Trisomy 21 causes an increase in DNA damage in neurons generated from iPSCs.
A: Neurons were generated from iPSCs over a 60 day differentiation protocol. Cells were then fixed and stained with a γH2AX antibody to detect DNA double-strand breaks. Nuclei were labelled with Hoechst. Scale bar: 100 μm. Enlarged insets for D21C3 and T21C5 double stained with Hoechst and γH2AX antibody are shown as examples below main images. B: The number of γH2AX puncta per cell is significantly increased in T21 neurons compared to the isogenic D21 neurons. Image capture and quantification was performed using automated multi-parametric analysis on the ImageXpress Micro XL (Molecular Devices) wide-field high content imaging system, and data analyzed using MetaMorph software. Three wells per cell line and a minimum of 6,000 cells per well were analyzed. Student's T-test, error bars SEM.

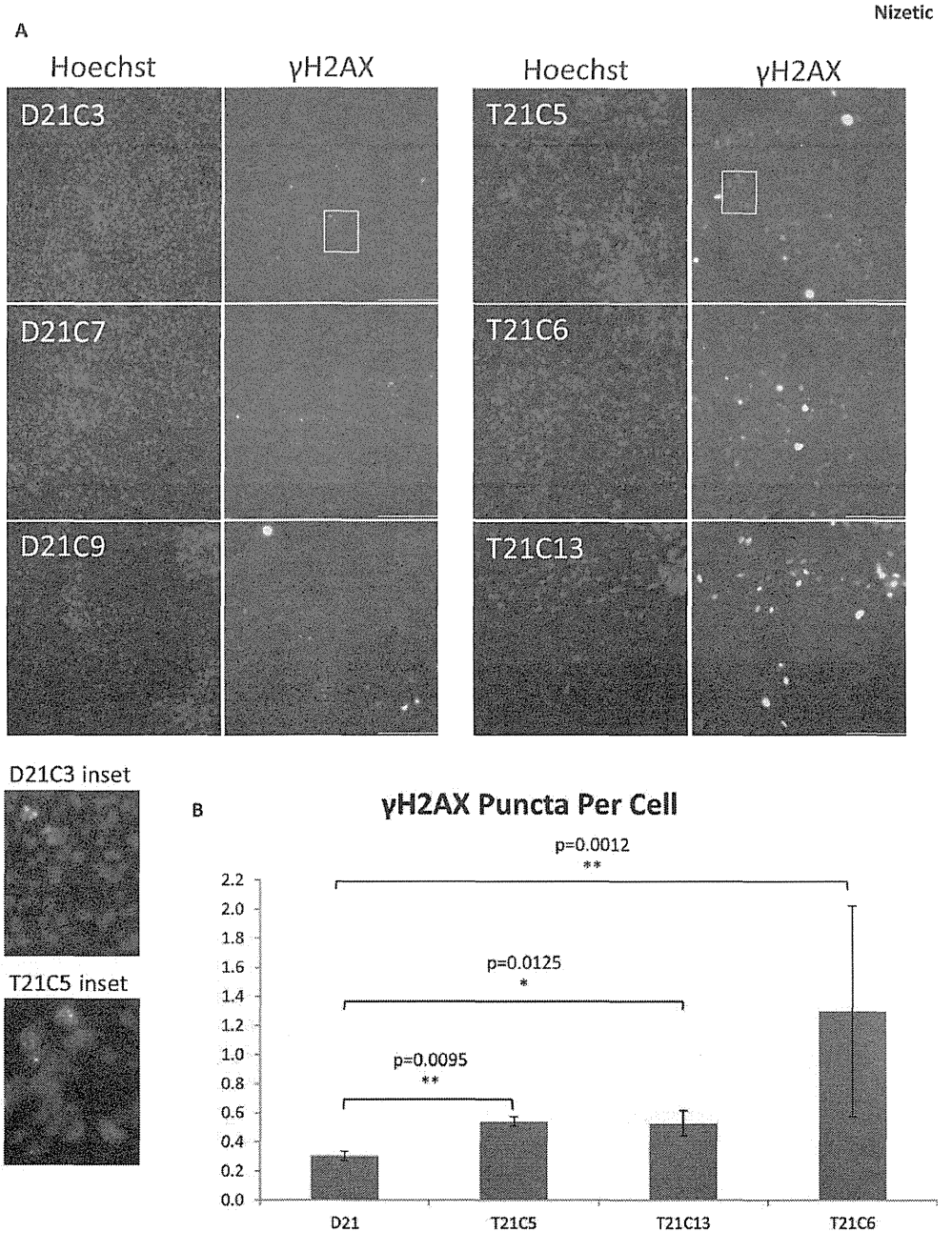


Fig. 4

Pediatr Transplantation 2015: 19: E66–E69

Pediatric Transplantation
DOI: 10.1111/petr.12447

Living donor liver transplantation from a heterozygous parent for classical maple syrup urine disease

Kadohisa M, Matsumoto S, Sawada H, Honda M, Murokawa T, Hayashida S, Ohya Y, Lee K-J, Yamamoto H, Mitsubuchi H, Endo F, Inomata Y. (2015) Living donor liver transplantation from a heterozygous parent for classical maple syrup urine disease. *Pediatr Transplant*, 19: E66–E69. DOI: 10.1111/petr.12447.

Abstract: MSUD is a hereditary metabolic disorder that is characterized by impaired activity of the BCKADC. Liver transplantation has been approved as a treatment for some MSUD cases in which the control of BCAAs is insufficient. Although there have been several reports about DDLT for MSUD, few LDLT cases have been reported. Because either of parents who are heterozygote of this disease usually applies to be a candidate of donor in LDLT, the impairment of BCKADC activity of graft liver should be concerned. We performed LDLT for 10 month-old girl with a left lateral segment graft from her father. BCKADC activities of the patient and her parents were measured using lysates of lymphocytes isolated from peripheral blood specimen before the transplant. As a consequence, the activity of BCKADC of father was not inferior to a normal range. The patient tolerated the operation well. Postoperative course was uneventful and mixed milk was started at 8th POD. The serum BCAAs levels have remained within normal range. It should be necessary to follow the physical growth and mental development of the recipient in the future.

Masashi Kadohisa[1], Shirou Matsumoto[2], Hirotake Sawada[3], Masaki Honda[1], Takahiro Murokawa[1], Shintaro Hayashida[1], Yuki Ohya[1], Kwang-Jong Lee[1], Hidekazu Yamamoto[1], Hiroshi Mitsubuchi[2], Fumio Endo[2] and Yukihiro Inomata[1]

[1]Department of Transplantation and Pediatric Surgery, Kumamoto University, Kumamoto, Japan, [2]Department of Pediatrics, Kumamoto University, Kumamoto, Japan, [3]Department of Pediatrics, Miyazaki University, Miyazaki, Japan

Key words: hereditary metabolic disorder – autosomal recessive – heterozygote – branched-chain alpha-keto acid dehydrogenase complex – branched-chain amino acids

Masashi Kadohisa, Department of Transplantation and Pediatric Surgery, Kumamoto University, Honjo 1-1-1, Chuo-ku, Kumamoto-shi, Kumamoto 860-8556, Japan
Tel.: 181 96 373 5616
Fax: 181 96 373 5783
E-mail: 0326mk@fc.kuh.kumamoto-u.ac.jp

Accepted for publication 23 January 2015

MSUD is an autosomal recessive metabolic disorder characterized by impaired activity of the branched-chain alpha-keto acid dehydrogenase complex (BCKADC). It leads to the accumulation of BCAAs and branched-chain alpha-keto acids. From early stage after birth, patients present the symptoms such as poor suckling, milk vomiting, apnea, convulsion, ketoacidosis, and so on, and psychosomatic growth is also affected. The early diagnosis and giving low BCAA milk is a basic treatment policy. The prognosis is relatively good by appropriate treatment. However, the risk of chronic cognitive impairment, mental illness, and sudden death cannot be avoided in some occasions under the nutritional control (1).

Liver transplantation has been approved as one of the treatments for MSUD, when the patient is difficult to be controlled for BCAAs with designed diet. Since the first report of the liver transplant for the MSUD was published in 1994 (2), there have been several reports about DDLT for MSUD and they have produced almost positive results (3). However, so far, few LDLT cases have been reported (4).

In Japan, as the number of DDLT has not increased so much, LDLT has still been a major modality. The problem in case of autosomal recessive heredity disease for living donation is that both parents are heterozygote. Because either of parents is usually selected as a donor candidate in LDLT, impairment of BCKADC activity of the graft liver in the heterozygote should be concerned. Although there are some

Abbreviations: BCAAs, branched-chain amino acids; BCKADC, branched-chain acid dehydrogenase complex; DDLT, deceased donor liver transplantation; LDLT, living donor liver transplantation; MSUD, maple syrup urine disease; PBMC, peripheral blood mononuclear cells; POD, postoperative day.

reports of LDLT from heterozygote in other autosomal recessive heredity diseases (5), feasibility of the heterozygous living donor has not been well described so far in the MSUD.

Case report

Patient was a 10-month-old girl. At gestational age of 38 wk and one day, she was delivered via cesarean operation because of breech presentation. There was no positive hereditary history related to congenital metabolic disorders in her family members.

As a result of mass screening submitted at five days old, leucine was 15.5 mg/dL and the blood gas analysis showed metabolic acidosis with respiratory compensation. As a result of tandem mass spectrometry at eight days old, leucine (35.0 mg/dL), valine (10.3 mg/dL), leucine + isoleucine (33.9 mg/dL) were high, and therefore, MSUD was diagnosed.

By diet management using the BCAAs removal milk and control of total calories, reduction in blood leucine, isoleucine, valine levels was tried. However, BCAA level was difficult to be controlled and stabilized well. When even one kind of amino acid became insufficient, weight gain became poor, and when even one kind of amino acid became excess in supply, she developed metabolic acidosis. Because of the difficulty in the medical control, liver transplantation was considered.

Because of the limited deceased donor availability in Japan, LDLT from one of the parents was considered. BCKADC activities of the patient and her parents were measured using lysates of lymphocytes isolated from peripheral blood specimen (6). Mother showed a lower activity than normal control group, but father's activity was not inferior to a normal range (Table 1). Each parent was willing to be a donor, but father was judged theoretically as the better donor. Because the father showed some hepatic steatosis at the first examination, in the re-assessment by liver biopsy after three months of diet, father was finally judged as the suitable donor.

Table 1. Mother showed a low activity than a normal control group; however, father's activity was not inferior to a normal control group

	BCKADC activity (pmol isovalery-CoA/min/10^6 cells)			
Subject Patient	1st Lower than sensitivity	2nd Lower than sensitivity	3rd Lower than sensitivity	Average Lower than sensitivity
Father	7.0	6.8	5.8	6.5
Mother	2.5	3.6	2.5	2.9
Control 1	6.2	4.4	6.9	5.8
Control 2	4.1	4.9	4.5	4.5
Normal average	5.7 ± 1.8			

Domino transplant was considered and the potential recipients (two recipients including the possible backup one) had been selected. However, just before the scheduled transplant, one donor died, and the other declined due to the social problem. Because the LDLT for the MSUD patient has the priority, the domino transplant in this case was canceled.

The patient underwent LDLT using a left lateral segment graft from her father. Operation time was 12 h, 23 min. Graft was 203 g in weight. Graft to recipient weight ratio was 2.69% (recipient body weight at the transplant was 7545 g). Cold ischemic time was 74 min and warm ischemic time was 45 min. The patient tolerated the operation well except for an episode of severe acidosis probably due to a stress of operation. Acidosis was spontaneously corrected immediately after the implantation of the graft, and it did not cause serious problems thereafter.

Postoperative course was uneventful in the early period. The liver function improved smoothly. However, after seven wk, she began to suffer from repeated vomiting. Adhesive intestinal obstruction was suspected, and she underwent relaparotomy and bowel resection with enteroclysis for this at 55th POD.

In terms of nutrition, mixed material of BCAAs removal milk and the common formula milk was started at 8th POD. The serum BCAAs levels have remained within normal range after that (Fig. 1). Preoperative daily natural protein intake was set as 0.46 g/kg/day. At six months after the transplant, daily natural protein intake has been gradually increased to 1.1 g/kg/day by increasing the amount of the mixed material and the proportion of the common formula milk (Fig. 2). As far as leucine goes, the tolerance increased from 50 to about 164 mg/kg/day according to an estimate by leucine content of the milk.

Discussion

Modality of treatment for the MSUD is still controversial in selection of liver transplantation or continued medical treatment. In previous reports about the intelligence prognosis after a liver transplantation for MSUD, the new liver did not restore the mental impairment (preoperative IQ <70) but did prevent further mental deterioration (3). The neurological outcome in MSUD is affected by quality of long-term metabolic con-

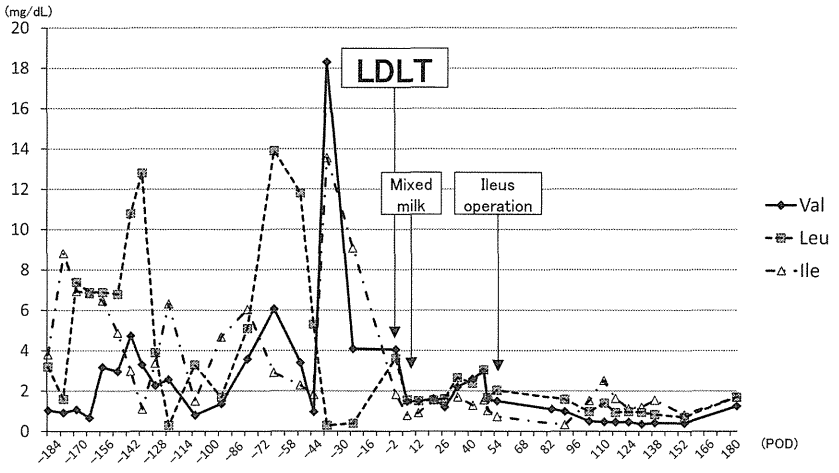


Fig. 1. Mixed material of the BCAAs removal milk and the common milk was started at 8th POD. In comparison with preoperative date, the serum BCAAs levels have remained within nearly normal range.

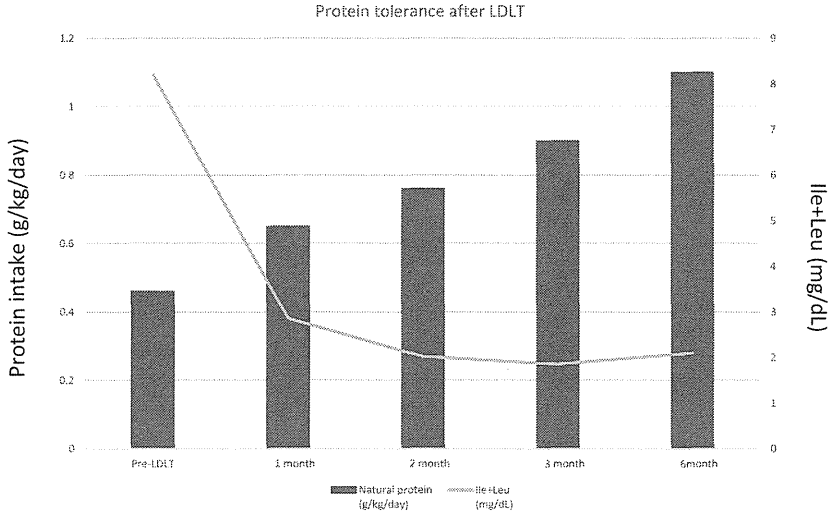


Fig. 2. The protein tolerance increased from 0.46 to 1.1 g/kg/day at six months after the transplant.

trol and variation in BCAAs levels in plasma (7, 8), especially during the period of rapid brain growth of infancy (9). Therefore, concerning the life and mental prognosis, when the medical treatment is difficult and BCAA levels tend to be unstable, liver transplantation should be considered not too late.

The number of DDLT is still limited in number in Japan, and the donated liver is allocated to more life-threatening patients. That is why patients with a congenital metabolism disease with some alternative treatments have to wait their turns for DDLT for a long time. Therefore, it is expected that the LDLT for the congenital metabolic disease such as MSUD will continue to play a further important role in the future.

BCKADC is expressed and metabolically active in liver, muscle, heart, and so on. Only 9–13% of BCKADC activity exists in the liver (10). Therefore, from a number of experiences of the past DDLT, it is expected that about 10% of normal BCKADC activity is sufficient to maintain blood amino acid homeostasis in unrestricted protein intake (11). In DDLT cases for the MSUD, it is thought that BCKADC activity of the liver graft is approximately normal. However, in LDLT from either of parents, theoretically lower activity of BCKADC in the graft liver would cause poor improvement of BCAA metabolism after the transplant. In the present case, we evaluated the BCKADC activity of parents in advance. There was no clear cutoff level fixed for discrimination of the heterozygote, and accuracy of the BCKADC activities measured using peripheral blood specimen was controversial. However, in comparison of the parents, father had higher BCKADC activity than mother, and the father was chosen as the donor.

In terms of donor selection, direct enzyme activity assay using the specimen taken by liver needle biopsy of the potential donor who was one of the heterozygous parents can be considered. However, needle biopsy should have more significant merit over the less harmful

examination to respect the donor safety in the setting of LDLT. In this case, using the lysates of PBMC lymphocytes, BCKADC activity of heterozygote was predicted 50%, and this was consistent with past reports. Considering the relatively small percentage of the defective enzyme in the liver in MSUD, we judged that 50% would be enough and that more specialized examination is unnecessary clinically.

The recipient has been doing well so far at the timing of this writing that is six months from the transplant. By assessment of the biochemical improvement of amino acid metabolism, LDLT using a liver graft from the heterozygous parent was quite effective. For the final evaluation of the usefulness of the LDLT from parents of the MSUD, however, long-term follow-up of her growth and development is essential.

Acknowledgment

We would like to thank Dr. Go Tajima (Department of Pediatrics, Hiroshima University Graduate School of Biomedical & Health Sciences) for providing BCKADC activity data.

References

1. Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited disease. Biochemistry (Moscow) 2002: 67: 611–612.
2. Netter JC, Cossarizza G, Narcy C, et al. Mid-term outcome of 2 cases with maple syrup urine disease: Role of liver transplantation in the treatment. Arch Pediatr 1994: 1: 730–734.
3. George VD, Holmes M, Rakesh S, et al. Liver transplantation for classical maple syrup urine disease: Long-term follow-up in 37 patients and comparative united network for organ sharing experience. J Pediatr 2012: 160: 116–121.
4. Feier FH, Miura IK, Fonseca EA, et al. Successful domino liver transplantation in maple syrup urine disease using a related living donor. Braz J Med Biol Res 2014: 47: 522–526.
5. Kasahara M, Sakamoto S, Horikawa R, et al. Living donor liver transplantation for pediatric patients with metabolic disorders: The Japanese multicenter registry. Pediatr Transplant 2014: 18: 6–15.
6. Tajima G, Yofune H, Bahagia Febriani AD, Nishimura Y, Ono H, Sakura N. A simple and rapid enzymatic assay for the branched-chain a-ketoacid dehydrogenase complex using high-performance liquid chromatography. J Inherit Metab Dis 2004: 27: 633–639.
7. Simon E, Schwarz M, Wendel U. Social outcome in adults with maple syrup urine disease (MSUD). J Inherit Metab Dis 2007: 30: 264.
8. Hoffmann B, Helbling C, Schadewaldt P, Wendel U. Impact of longitudinal plasma leucine levels on the intellectual outcome in patients with classic MSUD. Pediatr Res 2006: 59: 17–20.
9. Strauss KA, Wardley B, Robinson D, et al. Classical maple syrup urine disease and brain development: Principles of management and formula design. Mol Genet Metab 2010: 99: 333–345.
10. Suryawan A, Hawes JW, Harris RA, Shimomura Y, Jenkins AE, Hutson SM. A molecular model of human branched-chain amino acid metabolism. Am J Clin Nutr 1998: 68: 72–81.
11. Strauss KA, Mazariegos GV, Sindhi R, et al. Elective liver transplantation for the treatment of classical maple syrup urine disease. Am J Transplant 2006: 6: 557–564.

JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Effects of cyclodextrins on GM1-gangliosides in fibroblasts from GM1-gangliosidosis patients

Yuki Maeda[a,b], Keiichi Motoyama[a], Taishi Higashi[a], Yuka Horikoshi[c], Toru Takeo[c], Naomi Nakagata[c], Yuki Kurauchi[a,b], Hiroshi Katsuki[a], Yoichi Ishitsuka[a], Yuki Kondo[a], Tetsumi Irie[a,b], Hirokazu Furuya[d], Takumi Era[e] and Hidetoshi Arima[a,b]

[a]Graduate School of Pharmaceutical Sciences, [b]Program for Leading Graduate Schools 'HIGO (Health Life Science: Interdisciplinary and Glocal Oriented) Program', [c]Division of Reproductive Engineering, Center for Animal Resources and Development, [e]Department of Cell Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, [d]Department of Neurology, Kochi Medical School, Kochi University, Kochi, Japan

Keywords
cyclodextrins; fibroblasts; GM1-gangliosidosis; lysosomes

Correspondence
Hidetoshi Arima, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Chuo-ku, Kumamoto 862-0973, Japan.
E-mail: arimah@gpo.kumamoto-u.ac.jp

Received August 6, 2014
Accepted January 25, 2015

doi: 10.1111/jphp.12405

Abstract

Objectives GM1-gangliosidosis is an inherited disorder characterized by the accumulation of GM1-gangliosides in many tissues and organs, particularly in the brain. Currently, there is no treatment available for patients with ganglioside storage diseases. Therefore, we investigated the effects of cyclodextrins (CyDs) on the GM1-ganglioside level in EA1 cells, fibroblasts from patients with GM1-gangliosidosis.

Methods The concentrations of cholesterol and phospholipids in supernatants were determined by Cholesterol E-test Wako and Phospholipid C-test Wako, respectively. The effects of CyDs on GM1-ganglioside levels in EA1 cells using fluorescence-labelled cholera toxin B-subunit, which can bind to GM1-gangliosides specifically, were investigated by flow cytometry and confocal laser scanning microscopy.

Key findings The treatment with methylated CyDs, hydroxypropylated CyDs and branched CyDs decreased GM1-ganglioside levels in EA1 cells at 1 mM for 24 h. Unexpectedly, there was no significant change in the efflux of cholesterol or phospholipids from the cells after treatment with CyDs under the same experimental conditions, indicating that the efflux of membrane components is not associated with down-regulation of GM1-ganglioside levels in EA1 cells upon CyDs treatment.

Conclusions CyDs may have the potential as drugs for GM1-gangliosidosis, although the mechanism should be thereafter clarified.

Introduction

GM1-gangliosidosis is a rare lysosomal storage disorder characterized clinically by a wide range of variable neurovisceral, ophthalmological and dysmorphic features.[1] Without enough functional β-galactosidase which in humans is encoded by the *GLB1* gene, GM1-gangliosides cannot be degraded in lysosomes, and eventually GM1-gangliosides accumulate to toxic levels in many tissues and organs, particularly in the brain.[1] Several approaches for the treatment of GM1-gangliosidosis were developed, such as enzyme replacement therapy,[2] gene therapy,[3] chemical chaperone therapy.[4] However, at present, there is no treatment available for patients with ganglioside storage diseases.[5] Therefore, development of novel drugs for GM1-gangliosidosis is needed.

Cyclodextrins (CyDs) are cyclic oligosaccharides forming inclusion complexes with a wide range of hydrophobic molecules, and are used widely in pharmaceutical region.[6] Uekama and his colleagues previously reported that CyDs extracted cell membrane components such as cholesterol and phospholipids from lipid rafts, which contain high concentrations of cholesterol and glycosphingolipids including GM1-gangliosides.[7,8] Meanwhile, the administration of 2-hydroxypropyl-β-CyD (HP-β-CyD) to mice lacking Niemann-Pick disease type C (NPC) protein was reported

