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Modeling of Menkes disease via human induced pluripotent stem cells

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ABSTRACT

Menkes disease (MD) is a copper-deficient neurodegenerative disorder that manifests severe neurologic symptoms such as seizures, lethargic states, and hypotonia. Menkes disease is due to a dysfunction of ATP7A, but the pathophysiology of neurologic manifestation is poorly understood during embryonic development. To understand the pathophysiology of neurologic symptoms, molecular and cellular phenotypes were investigated in Menkes disease-derived induced pluripotent stem cells (MD-iPSCs). MD-iPSCs were generated from fibroblasts of a Menkes disease patient. Abnormal reticular distribution of ATP7A was observed in MD-fibroblasts and MD-iPSCs, respectively. MD-iPSCs showed abnormal morphology in appearance during embryoid body (EB) formation as compared with wild type (WT)-iPSCs. Intriguingly, aberrant switch of E-cadherin (E-cad) to N-cadherin (N-cad) and impaired neural rosette formation were shown in MD-iPSCs during early differentiation. When extracellular copper was chelated in WT-iPSCs by treatment with bathocuprione sulfate, aberrant switch of E-cad to N-cad and impaired neuronal differentiation were observed, like in MD-iPSCs. Our results suggest that neurological defects in Menkes disease patients may be responsible for aberrant cadherin transition and impaired neuronal differentiation during early developmental stage.

1. Introduction

Menkes disease (MD) is characterized as an inherited form of copper deficiency [1,2]. For this reason, Menkes disease has been studied for the past three decades as a disease model to understand the role of copper in the human nervous system [3]. Menkes disease is an infantile-onset X-linked recessive neurodegenerative disorder caused by dysfunction of a copper-transporting ATPase, ATP7A [4–6]. ATP7A dysfunction results in diminished copper uptake, thereby leading to a copper deficient state in a patient's body [7,8]. The clinical manifestation of Menkes disease reflects decreased activities of enzymes that require copper as a cofactor, including dopamine-β-hydroxylase, cytochrome c oxydase, and peptidylglycine-α-amidating monooxygenase [9]. Among a wide spectrum of clinical manifestations caused by dysfunction of copper-requiring enzymes, neurologic symptoms are closely related with mortality and morbidity in Menkes disease patients. Infants born with Menkes disease begin to exhibit failure to thrive and developmental delay within several months after birth. Affected infants gradually manifest neurologic impairments (e.g. hypotonia, lethargic states and seizures), and usually die within 3 years of birth [10]. Treatment of choice, intravenous copper histidine injection, has shown to reduce the seizure susceptibility and hypertonicity in some patients [11,12]. However, even under ideal circumstance, Menkes disease infants treated with copper histidine exhibits suboptimal clinical outcomes [10]. To understand the pathophysiology of Menkes disease, ATP7A knockout mouse models have been used [13–15]. ATP7A knockout mice showed deficiency of olfactory sensory neuronal maturation during early neuronal development [13]. Expression of genes involving myelination, energy metabolism and translation was downregulated in cerebral cortex and cerebellum tissues of a Menkes disease patient [16]. Despite various pathologies in neuronal tissues in knockout mice and human cadavers, how the copper-deficient environment caused by ATP7A mutation affects neurologic manifestation in Menkes disease patients is poorly understood. Recently, it has been reported that human induced pluripotent stem cells (iPSCs) are useful systems to study mechanisms on human diseases during early development in organogenesis because of their differentiation capability into diverse cell types [17,18]. Here, we found that MD-iPSCs exhibited an aberrant switch of E-cad to N-cad and abnormal neural rosette formation during early differentiation.

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Furthermore, MD-iPSCs were impaired in the structural integrity, including membrane recruitment and microdetachment, in the cell to cell junction. Knockdown of ATP7A expression did mimic cellular phenotypes of MD-iPSCs. Our findings demonstrate molecular and cellular aberrancies in MD-iPSCs. This study provides novel insights for understanding neurological pathophysiology in Menkes disease.

2. Materials and methods

2.1. Clinical history of a Menkes disease patient

We obtained fibroblasts from a 5 year old boy that was diagnosed as Menkes disease at 4 months after birth. Menkes disease patient's fibroblasts were obtained from Asan Medical Center under a protocol approved by the institutional review board. The patient had severe neurologic symptoms such as lethargic state and seizure (Table 1). Detailed clinical data were previously published [19]. He had a defective *ATP7A* gene in which G was changed to A on 4005th nucleotide of cDNA sequence (Fig. 1A).

2.2. Generation of induced pluripotent stem cells (iPSC) from a Menkes disease patient

Human iPSCs were generated from human skim fibroblasts (CRL-2097) and dermal fibroblasts of a Menkes disease patient by ectopic expression of OCT4, SOX2, KLF4, and C-MYC as previously described [20]. They were maintained on Mitomycin C (Sigma–Aldrich, St. Louis, MO)-treated mouse embryonic fibroblasts in the ESC medium at 37 °C. The embryonic stem cell medium consisted of DMEM-F12 (Invitrogen, Carlsbad, CA) supplemented with 20% serum replacement (Invitrogen), 1% NEAA (Invitrogen), 1% Penicillin–streptomycin (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma–Aldrich), and 4 ng/ml bFGF (Invitrogen).

2.3. Embryoid body (EB) formation and neuronal differentiation

Human iPSC colonies were divided into small pieces of approximately 5.5 mm × 5.5 mm squares by using McIlwain tissue chopper (Mickle Engineering, Westbury, NY), and then treated with 10 mg/ml dispase (Invitrogen) for 5 min to detach. Detached clumps were cultured in bFGF-free embryonic stem cell medium containing 10% SR for 7 days. Suspended EBs were attached on Matrigel™ (BD Bioscience, Bedford, MA)-coated dishes and then cultured in the same medium for 7 days. For neural rosette formation, spontaneous differentiation and directed neuronal differentiation were used as depicted in Supplementary data 3. For spontaneous differentiation, EBs were cultured for 7 days and attached on Matrigel-coated dishes. Attached EBs were cultured in the same medium for 7 days. For directed differentiation, we cultured EBs for 4–5 days and attached to Matrigel-coated dishes. Then, the cells were cultured in neuronal differentiation medium (DMEM/F12 + 20 ng/ml bFGF + N2 supplement) for 5–7 days.

Table 1
Clinical and molecular datas from Menkes disease with ATP7A mutation.

Family data	Age	5 years
	Sex	M
Clinical data	Initial onset	4 months
	CNS	Seizure, lethargic state
	NM	Hypotonia
	Other	Brittle hair
Genetic data	Nucleotide chages	c4005 + G > A
	Protein alteration	Exon 20 skipping

NM = neuromuscular.

2.4. RNA isolation and real time PCR analysis

Total RNAs were extracted from cells using the TRIzol Reagent (Invitrogen), and reverse-transcribed using M-MLV Reverse Transcriptase (Enzynomics, Daejeon, Korea) according to the manufacturer's protocol. Relative expression levels of genes were measured by real-time RT-PCR using 2× Prime Q-Master Mix (GENET BIO, Seoul, Korea) and analyzed with an iCycler iQ5 Real-Time detection system (Bio-Rad Laboratories, Hercules, CA). The primers used are listed in Supplementary Table 1. The reaction parameters for real-time RT-PCR were 95 °C for 10 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, and a final elongation step at 72 °C for 5 min. For comparative analyses, mRNA expression levels were normalized to *GAPDH* and then expressed as fold-change. The sample ΔCt (SΔCt) value was calculated from the difference between the Ct values of *GAPDH* and the target genes. The relative gene expression levels between the sample and control were determined using the formula $2^{-(S\Delta Ct - C\Delta Ct)}$.

2.5. Immunocytochemistry

Cells were grown on a 4-well cell culture slide (SPL lifescience, South Korea), fixed with 4% formaldehyde at 4 °C for 30 min, permeabilized with 0.1% triton X-100 in PBS, and blocked with 4% normal donkey serum (Cell Signaling Technologies, Beverly, MA) or 3% BSA (Sigma–Aldrich) for 1 h at room temperature (RT). Subsequently, antibodies against E-cadherin (1:100), N-cadherin (1:100), NESTIN (1:200), SOX2 (1:200), TUJ-1 (1:200), MAP2 (1:200) were incubated with the prepared cells at 4 °C overnight. Finally, cells were washed several times with PBST (0.1% Tween-20 in PBS) and incubated with Alexa Fluor 488- or cy3-conjugated secondary antibodies (Invitrogen). Fluorescence was analyzed using fluorescence microscope (Olympus, Japan) or a Zeiss LSM 510 confocal microscope (Carl Zeiss, Germany). Number of N cadherin$^+$/Sox2$^+$ neural rosettes were counted per each well and statistically analyzed by a Mann–Whitney test.

2.6. Western blot analysis

Harvested cells were re-suspended in EBC lysis buffer (50 mM Tris–HCL8.0, 300 mM NaCl, 0.5% NP40) containing 100 μg/ml lysozyme, 10 μg/ml aprotininm and 10 μg/ml leupeptin. (Sigma–Aldrich). The cells in suspension were lysed by three–five cycles of sonication for 1 s on ice. After sonication, lysates were centrifuged at 16,100×*g* for 5 min at 4 °C. Protein concentration was determined by using Brad-ford assay. All sample preparations were diluted in 1× SDS loading buffer (60 mM Tris–HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol, 0.1% bromphenol blue) and boiled for 2–3 min. Proteins were separated on 10% SDS–PAGE gel, high molecular weight protein (over 100 kDa) were separated on 6% SDS–PAGE gel respectively, and then transferred to the nitrocellulose membrane. After blocking with 4% of skim milk or 5% BSA, membranes were incubated with antibodies at 4 °C overnight, respectively. After washing with TBST, membranes were incubated with horseradish peroxidase-conjugated second antibodies in TBST containing 4% skim milk for 1 h. Quantitative Imaging of blots by ECL chemi-luminescence was detected with Fujifilm LAS4000 CCD camera system (Fugifilm, Japan).

2.7. Antibodies and reagent

Primary antibodies used in this study were anti-vimentin rat Ab (R&D systems); anti-E-cadherin mouse Ab and anti-N-cadherin mouse Ab (BD Transduction Laboratories, Lexington, KY); anti-Snail and anti-slug rabbit Ab and anti-SOX2 rabbit Ab (Cell Signaling Technologies); anti-Nestin mouse Ab (Chemicon, Temecula,