신경계 질환 연구를 위한 환자유래 –유도만능줄기세포의 확립

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Establishment of a Patient-derived iPS Cell Line for the Study of Neurological Disorders

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This review showed that the patient specific iPSC made using skin cells can be a very useful tool for studying neurological disorders including Charcot-Marie-Tooth disease patients. In iPS production, skin biopsy tissues are mainly used, and iPSCs usually change four DNA molecules to recreate them into stem cells. Skin fibroblasts are the origin of ectoderm and neurons are the same origin of ectoderm. Because of this similarity, the iPSC produced using skin fibroblasts are easy to differentiate into neurons and works well. For this reason, iPSC production using skin tissue cells is useful for the study of neurological diseases, and it is possible to make good experimental results. iPSC can be used for treatment in two major ways; one is used for treat directly to the created iPSC into the patient and the other is using the iPSC for drug screening. The drug screening method is used in many cases because it can make an optimal in vitro study because it creates an environment similar to the patient. The findings in this review demonstrate in vitro drug screening models of inherited neuropathy resulting from mutations in the HSPB1 gene, can be developed from patient-specific iPSCs.

Key Words: Charcot-Marie-Tooth disease, Heat shock 27 kDa protein 1 (HSPB1), Induced pluripotent stem cells (iPSCs),

Introduction

Patient-derived induced pluripotent stem cell (iPSC) can be a very useful tool for studying neurological disorders. iPSCs usually change four DNA molecules to recreate them into stem cells. In iPSC production, skin biopsy tissues are mainly used. Skin fibroblasts are the origin of ectoderm and neurons are the same origin of ectoderm. Because of this similarity, the iPSC produced using skin fibroblasts are easy to differentiate into neurons and works well. For this reason, iPSC production using skin tissue cells is useful for the study of neurological diseases, and it is possible to make good experimental results. iPSC can be used for treatment in two major ways; one is used for treat directly to the created iPSC into the patient and the other is using the iPSC for drug screening. The drug screening method is used in many cases because it can make an optimal in vitro study because it creates an environment similar to the patient. In this review, iPSC is made using skin cells from Charcot-Marie-Tooth disease (CMT) patients, and the iPSC experiment is demonstrated through the use of it for the study of neurological diseases.

CMT is a heterogeneous group of neurodegenerative disorders. To date, over 80 genes have been identified as being responsible for these disorders.[1] CMT is mainly divided into demyelinating neuropathy (CMT1) and axonal neuropathy (CMT2) according to electrophysiological and histopathological features. Within these types, CMT type 2F

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(CMT2F) and distal hereditary motor neuropathy 2B (dHMN2B) are caused by heat shock 27 kDa protein 1 (HSPB1; also known as HSP27) mutation in chromosome 7q11.23.[2] The most frequent pathologies observed in CMT2F and dHMN2B are abnormal axonal transport and cytoskeleton organization.[3-5] However, our knowledge of the underlying molecular mechanisms is still limited, and specific therapies are not yet available.

Methods

Skin fibroblasts were obtained from skin biopsy (3 mm-punch) of control individuals and two patients carrying mutant forms of HSPB1 (S135F and P182L) with informed consent after Institutional Review Board approval. Fresh skin samples were minced and digested in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 mg/ml collagenase type IV, 50 U/ml dispase, and 0.05% trypsin/EDTA for 40 min at 37°C. After filtration through a cell strainer, cells were washed twice and harvested in DMEM containing 20% fetal bovine serum (FBS) and 100 μg/ml penicillin/streptomycin.

Four types of Sendai virus-containing vectors, each expressing different transcription factors (Sox2, Oct4, Klf4, and c-Myc; Invitrogen, Carlsbad, CA, USA), were introduced into fibroblasts derived from patients with a multiplicity of infection of 3 by following manufacturer's protocol. On the day 7, cells were trypsinized and transferred onto mitomycin C (Sigma, St. Louis, MO, USA)-treated mouse embryonic fibroblasts, SNL feeder cells (Cell Biolabs, Inc., San Diego, CA, USA), and harvested with embryonic stem cell (ESC)/iPSC medium (KnockOut™, Gibco, Grand Island, NY, USA) containing 4 ng/ml basic fibroblast growth factor (bFGF). The medium was changed daily. On day 30, iPSC colonies were selected based on their morphological characteristics. Other stem cells used as controls included human ESCs (WA09; WiCell, Madison, WI, USA) and human iPSCs (hFSiPS1; Korea National Institute of Health, Chungbuk, Korea).

CMT2F-iPSCs and dHMN2B-iPSCs were cultured without feeder cells at cellular passages of 10, and 20 cells from each type of iPSCs were analyzed for the karyoptype by Seegene Medical Foundation (Seoul, Korea).

Equal volumes of mixtures of Matrigel (Corning, Corning, NY, USA) and 1.0×10^6 human ESCs (WA09), CMT2F-iPSCs, or dHMN2B-iPSCs were subcutaneously injected into the backs of 5-week-old female NOD/SCID mice (Laboratory Animal Resource Center, Korean Research Institute of Bioscience and Biotechnology, Daejeon, Korea). The xenografts were allowed to grow for 8 weeks and were then explanted by surgical procedures. Teratoma tissues were fixed in 10% formaldehyde and embedded in paraffin. For histological analysis, hematoxylin and eosin staining was performed.

ESCs and iPSCs were plated onto ultra-low binding plates (i.e., uncoated Petri dishes) and cultured in suspension with ESC/iPSC medium (KnockOut™), which was replaced every other day. After 8 days in floating culture, EBs were transferred onto a gelatin-coated (Sigma) chamber slide (Nalgene/Nunc, Rochester, NY, USA) and cultured in 10% FBS/DMEM (Welgene, Daegu, Korea) to allow differentiation randomly into three germ-layered cells for an additional 8 days.

For the detection of total and endogenous expression of KLF4, OCT4, SOX2, and c-MYC, and HSPB1, RNA extraction from ESCs and CMT2F-iPSCs was performed with Trizol. Reverse transcription was performed using AMV reverse transcriptase (Promega, Madison, WI, USA), and PCR was performed using Ex Taq polymerase (Takara Bio, Otsu, Japan).

To generate EBs, colonies of ESCs and iPSCs were enzymatically dissociated into small clumps and cultured in suspension for 2 days in a Petri dish supplemented with ESC/iPSC medium (KnockOut™) containing 10 μM Rho-associated kinase inhibitor Y27632 (Tocris Bioscience, Bristol, UK), 20 ng/mL bFGF (Invitrogen), 10 μM SB435142 (Stemgent, Cambridge, MA), and 0.2 μM LDN193189 (Stemgent) and penicillin/streptomycin. On day 5, for

caudalization, retinoic acid (1 μM; Sigma), ascorbic acid (0.4 μg/ml; Sigma), brain-derived neurotrophic factor (10 ng/ml; R&D, Minneapolis, MN, USA), and N2 supplement (1%; Gibco) were added.[6] On day 7, for ventralization, the sonic hedgehog agonist purmorphamine (1 μM; Stemgent) was added, and the dual SMAD inhibitors (SB435142 and LDN193189) were discontinued. On day 17, basal medium was changed to neurobasal medium (Invitrogen) containing all previously indicated factors with the addition of insulin-like growth factor-1 (10 ng/ml), glial cell line-derived neurotrophic factor (10 ng/ml), and B27 (2%; Gibco). On day 21, neurospheres were dissociated with accutase (Gibco) and plated onto poly-L-lysine/laminin-coated culture plates or slide chambers (Nalgene) and supplemented with neurobasal medium containing all previously indicated factors with the addition of β-mercaptoethanol (25 μM; Gibco) and glutamic acid (25 μM; Sigma).

Neurospheres derived from ESCs and iPSCs were dissociated with accutase into single cells and seeded onto microchannel plates at a density of 1×10^5 cells/plate and cultured with Neurobasal (Invitrogen) and B27 medium for 10 days. After axons had completely stretched through the μm-sized grooves (total length = 833.4 μm) and reached the opposite compartment, MNs were transfected with mito-dsRED2 (Clonetech Inc./Takara Bio) delivered by lipofectamine 2000 (Invitrogen). Within 3 days of transfection, mitochondrial images were captured by a fluorescent microscope at a rate of 121 snaps/2 min and stacked into one file to create kymographs. Mitochondrial moving velocity was calculated by measured angle and distance in kymograph using ImageJ software. Axonal lengths were also measured using ImageJ. Axonal lengths were calculated by summation of measured length of axon stretched out from a single μm-sized groove and known length of the groove (833.4 μm).

Results and discussion

Dermal fibroblasts were biopsied from CMT2F patient (female/52-year-old, Korean) with 404 C>T (S135F) mutation and dHMN2B patient (female/8-year-old, Korean) with 545 C>T (P182L) mutation of the HSPB1 gene. Both of them showed predominant distal leg muscle weakness and toe gait abnormalities. The ages at onset were 25-year-old and 7-year-old, respectively. Sensory neve conduction velocities and action potentials of sural nerves were decreased in CMT2F patient, but within normal ranges in dHMN2B patient.

Fibroblasts were reprogrammed into iPSCs by Sendai viral transduction of four episomal vectors carrying KLF4, OCT3/4, SOX2, and c-MYC. The morphology of CMT2F-iPSC and dHMN2B-iPSC colonies, which resembled that of human ESCs (WA09) consisted of cells having a high nuclear-to-cytoplasm ratio compacted in a flat cobblestone-like appearance with sharp edges. The genetic background of CMT2F-iPSCs and dHMN2B-iPSCs was not changed during the reprogramming processes, especially at the mutation site of HSPB1. CMT2F-iPSCs and dHMN2B-iPSCs preserved their normal karyotype. The expression of endogenous KLF4, OCT3/4, SOX2, and c-MYC genes was detected by RT-PCR after a few sub-passages using primers with complementary sequences to the intron area of the target mRNA. Sendai viral genome contents were not detected in iPSCs after cellular passage of 10. CMT2F-iPSCs and dHMN2B-iPSCs expressed stem cell markers such as NANOG in the nucleus and SSEA in the cytoplasm. The pluripotency of CMT2F-iPSCs and dHMN2B-iPSCs was verified by the presence of randomly differentiated AFP-positive endodermal cells, SMA-positive mesodermal cells, and nestin-positive ectodermal cells via EB formation in vitro and teratoma formation in vivo.

To recapitulate peripheral neuropathy, MNs were differentiated from iPSCs by providing dual SMAD inhibitors (SB435142 and LDN193189) for neuralization, retinoic acid for caudalization, and purmorphamine for ventralization according to the method described by Amoroso. Fully differentiated MNs expressed transcription factors such as HB9 and ISL1/2, cytoskeletal markers such as Tuj1, MAP2, and

SMI32, and synapsin and ChAT. S135F-MNs and P182L-MNs showed no developmental defects, evidenced by no differences between S135F-MNs and P182L-MNs and control WA09-MNs and hFSiPS1-MNs in the proportion of marker-positive cells. WA09-MNs (1236 ± 23 μm) and S135F-MNs (1287 ± 20 μm) showed no differences in axonal length. Neuromuscular junctions, visualized by α-bungarotoxin staining, formed when MNs were co-cultured with myotube cells differentiated from C2C12 cells.

Although there is heterogeneity in causative genes for different CMT2 subtypes, many disease subtypes involve abnormalities in the cellular trafficking system. As MNs can have long axons up to one meter in length, defects in axonal transportation may increase vulnerability to axonopathy. In particular, mitochondrial transport is extremely important for maintaining axonal and synaptic stability in neurons. During bidirectional trafficking of mitochondria along microtubules, quality control is accomplished by dynamic fusion and fission processes that enable mitochondria to generate ATP to support vital cellular functions and buffer intracellular calcium. Therefore, we tested whether S135F-MNs and P182L-MNs have defects in mitochondrial axonal transport by culturing cells in micro-channel plates, which compartmentalize axons from soma and dendrites, transfecting cells with mito-dsRED2, and analyzing kymograph images. We observed that the absolute velocity of mitochondrial movements was significantly lower in S135F-MNs (0.19 ± 0.01 μm/sec) and slightly lower in P182L-MNs (0.22 ± 0.01 μm/sec) compared with control WA09-MNs (0.24 ± 0.01 μm/sec) and hFSiPS1-MNs (0.25 ± 0.01 μm/sec). Also the proportion of moving mitochondria was significantly decreased in S135F-MNs (26.37 ± 5.06%) and P182L-MNs (14.19 ± 2.14%) than those in control WA09-MNs (31.39 ± 3.74%) and hFSiPS-MNs.

Axonal transport is regulated by various post-translational modifications of microtubules through the recruitment of molecular motor proteins. Among these modifications, acetylation of α-tubulin at the protein site of K40 regulates binding of dynein/dynactin complexes and kinesin-1 to microtubules.[7] In addition, treatment with HDAC6 inhibitors, which increase acetylation of α-tubulin, reverses transport defects in cellular models of Huntington's disease and LRRK2 mutation-induced Parkinson's disease. Therefore, acetylation levels of α-tubulin were examined as an indicator of mitochondrial transport defects in S135F-MNs and P182L-MNs. We found that S135F-MNs and P182L-MNs showed a significant reduction in acetylated α-tubulin levels compared with WA09-MNs or hFSiPS1-MNs.

By the treatment with HDAC6 inhibitors, including tubastatin A (5 μM) and newly developed CHEMICAL X4 (0.5 μM and 5 μM) and CHEMICAL X9 (0.5 μM and 5 μM) for 3 hrs, acetylation of α-tubulin in S135F-MNs and P182L-MNs were increased. Moreover, the absolute velocity of mitochondrial movements and the proportion of moving mitochondria in axons were increased significantly by the treatment of these HDAC6 inhibitors.

Those results demonstrate in vitro drug screening models of inherited neuropathy resulting from mutations in the HSPB1 gene, can be developed from patient-specific iPSCs. Axonal pathologies were mimicked in microfluidic culturing system as both S135F-MNs and P182L-MNs showed a marked decline in the absolute velocity and the percentage of moving mitochondria. These axonal defects were associated with decreased acetylation of α-tubulin and were reversed by treatment of HDAC6 inhibitors

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