Established Stem Cell Model of Spinal Muscular Atrophy Is Applicable in the Evaluation of the Efficacy of Thyrotropin-Releasing Hormone Analog

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ABSTRACT

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by the degeneration of spinal motor neurons. This disease is mainly caused by mutation or deletion of the survival motor neuron 1 (SMN1) gene. Currently, no effective treatment is available, and only symptomatic treatment can be provided. Our purpose in the present study was to establish a human SMA-derived induced pluripotent stem cell (SMA-iPSC) disease model and assay a therapeutic drug in preparation for the development of a novel treatment of SMA. We generated iPSCs from the skin fibroblasts of a patient with SMA and confirmed that they were pluripotent and undifferentiated. The neural differentiation of SMA-iPSCs shortened the dendrite and axon length and increased the apoptosis of the spinal motor neurons. In addition, we found activated astrocytes in differentiated SMA-iPSCs. Using this model, we confirmed that treatment with the thyrotropin-releasing hormone (TRH) analog, 5-oxo-L-prolyl-L-histidyl-L-prolinamide, which had marginal effects in clinical trials, increases survival motor neuron protein levels by inhibiting glycogen synthase kinase (GSK)-3β activity. Finally, the TRH analog treatment resulted in dendrite and axon development of spinal motor neurons in differentiated SMA-iPSCs. These results suggest that this human in vitro disease model stimulates SMA pathology and reveal the potential efficacy of TRH analog treatment for SMA. Therefore, we can screen novel therapeutic drugs such as TRH for SMA easily and effectively using the human SMA-iPSC model.

SIGNIFICANCE

Platelet-derived growth factor (PDGF) has recently been reported to produce the greatest increase in survival motor neuron protein levels by inhibiting glycogen synthase kinase (GSK)-3β; however, motor neurons lack PDGF receptors. A human in vitro spinal muscular atrophy-derived induced pluripotent stem cell model was established, which showed that the thyrotropin releasing hormone (TRH) analog promoted transcriptional activation of the SMN2 gene and inhibition of glycogen synthase kinase-3β activity, resulting in the increase and stabilization of the SMN protein and axon elongation of spinal motor neurons. These results reveal the potential efficacy of TRH analog treatment for SMA.

INTRODUCTION

Spinal muscular atrophy (SMA) is an inherited autosomal recessive disease characterized by a progressive loss of motor neurons in the anterior horn of the spinal cord, which leads to skeletal muscle weakness and atrophy [1]. Clinically, SMA is classified as type O to type IV, according to the severity, age of onset, and achievement of physical development [2, 3]. SMA type 0 is the most severe type, the onset is prenatal, and death occurs within weeks without support. SMA type I is severe, the onset is within 6 months of age, and exercise development stops after onset. SMA type II is intermediate in severity, with onset within 18 months of age and the patient is unable to stand without support. SMA type III is mild in severity, with onset after 18 months of age and the patient becomes unable to stand gradually as the disease progresses. SMA type IV is the mildest, with adult onset, with motor function declining slowly. These forms of SMA are mainly caused by low levels of the survival motor neuron (SMN) protein, which is ubiquitously expressed and coded by two genes, SMN1 and SMN2.
and SMN2 [4, 5]. In most patients with SMA, the SMN1 gene is deleted or mutated [6], and the SMN protein is produced in reduced amounts by the SMN2 gene [7]. The SMN2 gene has several copies and is associated with the clinical severity of SMA. The severe form of SMA is usually associated with one to two copies, the intermediate form with two to three copies, and the mild or adult form with three to four or even five to six copies [8, 9].

Currently, no curative agents are available for SMA, although some research groups have developed treatments based on the molecular pathophysiology of this disease [10]. The current treatment strategies can be classified into three major groups [10], including SMN2 targeting [11, 12], SMN1 introduction [13, 14], and non-SMN targeting [15, 16]. Many of these treatments have shown therapeutic potential in patient-derived fibroblast-based assays or preclinical animal models. However, all clinical trials reported to date have failed to show significant effectiveness of these therapeutic approaches [10]. The lack of in vitro human neuronal cell disease models has contributed to the misidentification of a clinically effective treatment for this disease. Therefore, such a model could be a potential source of an effective treatment strategy. Recently, a drug assay and development systems using a patient-derived induced pluripotent stem cell (iPSC) disease model have been reported [17, 18]. In these studies, docosahexaenoic acid treatment showed the alleviation of the stress responses in neurons differentiated from Alzheimer’s disease patient-derived iPSCs, and statin treatment showed the correction of the degraded cartilage in both chondrogenically differentiated thanatophoric dysplasia type I patient-derived and achondroplasia patient-derived iPSCs. Additional drug assay and developmental studies with this system have been eagerly envisaged worldwide, in particular, those using not easily available cell types such as neurons.

In the present study, we report on the human in vitro disease model developed by generating disease-specific iPSCs derived from a patient with SMA type III (human in vitro SMA-iPSC model). This patient was treated with an intravenous thyrotropin-releasing hormone (TRH) analog, 5-oxo-L-prolyl-L-histidyl-L-prolinamide, which we had previously tested in SMA clinical trials [19]. The walking cadence and speed of this patient were improved. However, the mechanism of action of the TRH analog in SMA remains unclear [10], although it has been suggested to improve metabolism and stimulate or protect neuronal cells in SMA patients. Our model revealed that the TRH analog increased the level of SMN2 transcription and inhibited glycogen synthase kinase (GSK)-3β, thereby increasing SMN protein levels in the spinal motor neurons differentiated from the SMA-iPSCs. Accordingly, the TRH analog enhanced dendrite and axon development in the SMA-iPSC-derived spinal motor neurons. This model can provide a useful drug development system for screening effective treatment approaches.

The pathological analysis and establishment of patient-derived iPSCs (including human gene analysis research) in the present study were approved after review by the Ethical Review Committee of the National Hospital Organization, Nagara Medical Center. During the present study, the established human stem cells were handled according to the Revisions of the Guidelines for Clinical Research using Human Stem Cell from the Ministry of Health, Labor, and Welfare of Japan.

**Clinical Trial**

Our patient in the present study was tested as described previously [19]. In brief, the therapeutic dose (0.1 mg/kg daily) of the TRH analog (protirelin) was administered for 20 days. Vital signs and pulse oximetry were monitored for 3 hours after administration of the medication each day. The patient was also monitored to ensure that no toxic or undesirable effects, such as flushing, nausea, or abdominal discomfort, were observed during the therapy. In addition, laboratory tests, including determination of the levels of serum thyroid-stimulating hormone (TSH), were performed. The effect of TRH analog therapy was determined using video-based methods and motion capture data-driven methods. Three-dimensional motion capture analysis was used to quantitatively evaluate the effect [20]. In brief, our patient wore markers near each joint, and her motion was recorded by the positions or angles between the markers. These records were analyzed from three independent movements before therapy and after 20 days of therapy. The data are presented as the 95% confidence intervals. The statistical significance of the data was analyzed using the two-tailed paired t test. A p value < .05 indicated statistical significance.

**iPSC Culture and Spinal Motor Neuron Differentiation**

The iPSC colonies were differentiated using primate embryonic stem (ES) cell medium (ReproCELL, Kanagawa, Japan, http://www.reprocell.com) supplemented with 4 ng/ml basic fibroblast growth factor (Wako, Osaka, Japan, http://www.wako-chem.co.jp) and 500 U/ml penicillin/streptomycin (PS; Life Technologies, Carlsbad, CA, http://www.lifetechnologies.com). The iPSC colonies were cultured in 5% CO₂ at 37°C and passaged every 7 days. In the present study, we adopted a previously reported modified protocol that mimics normal motor neuron generation [17, 21–23]. In the first stage, we used a serum-free floating culture of embryoid body (EB)-like aggregates with the quick reaggregation (SFEBq) method, as described previously [17, 21]. We treated 5,000 single iPSCs with 2 µM dorsomorphin (Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com), 10 µM SB431542 (SB; Cayman Chemicals, San Diego, CA, http://www.caymanchem.com), and 10 µM p-associated coiled-coil forming kinase inhibitor, Y-27632 (Wako). This treatment was applied in a differentiation medium containing Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Life Technologies), 5% knockout serum replacement (Life Technologies), and 500 U/ml PS for the first 2 days. Then, the medium was changed to the first differentiation medium containing 2 µM dorsomorphin and 10 µM SB and changed every 2 or 3 days. Seven days after neural induction using the SFEBq method, the aggregates were transferred onto Matrigel-coated 96-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com) and treated with 2 µM dorsomorphin and 10 µM SB in the first differentiation medium for another 7 days. After induction, the neural precursor cells were

**MATERIALS AND METHODS**

**Ethics Statement**

The ethical committee of the Gifu University Hospital approved the clinical study of intravenous TRH analog therapy, and the parents of our pediatric patient provided informed consent. The study was performed in accordance with the provisions of the Ethical Guidelines for Clinical Studies of the Ministry of Health, Labor, and Welfare of Japan.
was changed every 2 or 3 days. For the GFAP- and HB9-positive cells assay, we counted the cells that were positive for these markers and Hoechst 33342 staining was performed using Hoechst 33342 (Life Technologies). The primary antibodies used are listed in supplemental online Table 1, and the secondary Alexa Fluor 488-labeled antibodies used included 594 donkey anti-rabbit, 594 donkey anti-goat IgG, 594 donkey anti-mouse IgG, 488 donkey anti-mouse IgG, 488 donkey anti-rabbit IgG, and 488 donkey anti-goat IgG (Life Technologies) for 15 minutes followed by three washings. The cells were then washed and incubated with the Alexa Fluor 488 donkey anti-rabbit IgG (Life Technologies) for 15 minutes followed by three washings.

Fluorescence-Activated Cell Sorting Analysis

Cell clumps were harvested using Accutase (Innovative Cell Technologies Inc., San Diego, CA), and gently dissociated into single cells, which were washed with a fluorescence-activated cell sorting (FACS) buffer containing 0.1% PBS, sodium azide (Wako), and 2% donkey serum. The cells were treated with Cytofix/Cytoperm fixation and permeabilization solution (Becton, Dickinson and Company) for 20 minutes at 4°C and incubated overnight with the anticleaved caspase 3 antibody (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com). The cells were then washed and incubated with the Alexa Fluor 488 donkey anti-rabbit IgG (Life Technologies) for 15 minutes followed by three washings. The cells were analyzed using a BD FACS Canto II (Becton, Dickinson and Company), and the ratio of cleaved caspase 3-positive cells was determined.

The data are presented as the mean ± SEM. The statistical significance of the data was evaluated using the two-tailed Student t test. A p value < .05 indicates statistical significance.

Reverse Transcription-Polymerase Chain Reaction and Quantitative Real-Time Polymerase Chain Reaction

The motor neurons were cultured for the appropriate period, dissolved in Isogen (Wako), and stored at −80°C until the RNA was extracted. The protein was removed using chloroform solution (Wako), and RNA was extracted with 2-propanol (Wako) and 70% ethanol (Wako). The concentration of RNA extracted was determined spectrophotometrically in a NanoView Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). First-strand cDNA was synthesized from 1 μg of total RNA using ReverTra Ace (Toyobo, Osaka, Japan, http://www.toyobo-global.com). The cDNA samples were subjected to polymerase chain reaction (PCR) amplification using a thermal cycler 2720 (Applied Biosystems, Carlsbad, CA, http://www.appliedbiosystems.com). The PCR was performed using Ex-Taq (Takara, Otsu, Japan, http://www.takara.co.jp) with the following cycles: for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), initial denaturation at 94°C for 2.5 minutes, followed by 25 cycles of 94°C for 30 seconds, 60°C for 1 minute, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. For the other genes, the cycles consisted of initial denaturation at 94°C for 2.5 minutes, followed by 30–40 cycles of 94°C for 30 seconds, 58°C–62°C for 60 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. The sequence of these gene primers is shown in supplemental online Table 2, and the human spinal cord total RNA (catalog no. 636554; Clonetech Laboratories Inc., Mountain View, CA) was used as the positive control sample.

The quantitative real-time PCR (qPCR) assay was performed using power SYBR green PCR master mix (Applied Biosystems) containing 250 nM FL-SMN, Δ7-SMN, and H89
Therapeutic Effect of the TRH Analog in a Clinical Study

Our patient was a 4-year-old girl diagnosed with SMA type III. She had shown normal development until the age of 18 months, when she could walk alone but experienced frequent falls while walking. She was diagnosed with SMA type III from the clinical symptoms, and genetic studies revealed that she exhibited the homozygous deletion of the SMN1 gene.

She was admitted to the Gifu University Hospital for inpatient TRH analog (protirelin) therapy, which was injected daily with 1.4 mg (0.1 mg/kg daily) for 20 days. She showed only minor transient adverse effects, including flushing, nausea, and abdominal discomfort, during the treatment. These adverse effects developed after several minutes of the injection, but her symptoms were mild. TSH was transiently suppressed (before therapy, 2.43 ng/ml; during therapy, average 1.24 ng/ml; and after therapy, 2.05 ng/ml), and no other abnormalities were found in her blood chemistry laboratory analysis or serum levels of pituitary hormones. After the TRH analog therapy, she was able to walk more easily and faster than before without falling, as determined from repeated video monitoring. In addition, motion capture analysis was performed. The kinetic analysis showed that her walking parameters had improved after therapy, including gait cadence (before, 112.9 ± 7.16 steps per minute and after, 136.9 ± 5.4 steps per minute), walking speed (before, 0.69 ± 0.12 m/s and after 0.95 ± 0.05 m/s), and gait stride length (before, 0.73 ± 0.06 m and after, 0.83 ± 0.020 m), indicating a clinical benefit (Fig. 1). In particular, her gait cadence and walking speed had significantly improved after therapy.

Characterization of iPSCs Derived From SMA Patients

We generated iPSCs derived from the FbCs of our patient with SMA type III and determined the effects of the TRH analog on the spinal motor neurons. Initially, the patient’s dermal FbCs (SMA-FbCs) were generated, followed by generation of the iPSCs using the episomal vector reprogramming factor (OCT3/4, SOX2, KLF4, LIN28, L-MYC, and p53 shRNA) [24] (Fig. 2A). The SMA-iPSCs exhibited a normal karyotype (Fig. 2B). Reverse transcription-PCR (RT-PCR) analysis revealed that SMA-FbCs and iPSCs had lower levels of the full-length transcripts (FL-SMN), including SMN1 and SMN2, and higher levels of transcripts of SMN2 lacking exon 7 (∆7-SMN), compared with FbCs and iPSCs from a control individual (Fig. 2C). In addition, when FL-SMN and ∆7-SMN products were digested with Dde1, specifically cleaving SMN2, the loss of SMN1 was confirmed in SMA-FbCs and iPSCs (Fig. 2C). These results revealed that our patient had SMA and generated iPSCs were derived from our patient who was administered intravenous TRH analog therapy.

Next, we examined whether the generated SMA-iPSCs had similar characteristics to human ES cells. We confirmed that the generated iPSCs were positive for ALP staining (Fig. 2D). In addition, we performed immunostaining using the undifferentiated markers and confirmed that OCT3/4, SOX2, and NANOG were expressed in the nuclei of generated iPSCs, and SSEA4, TRA-1-60, and TRA-1-81 were expressed in the cytoplasm (Fig. 2D). The generated iPSCs did not express SSEA1, which is a mouse undifferentiated marker (Fig. 2D). In addition, we confirmed the pluripotency of SMA-iPSCs using EB formation, which stained the ectoderm, endoderm, and mesoderm with markers of TUJ1, SOX17, and BRACHYURY, respectively (Fig. 2E).

Induction of iPSCs to Spinal Motor Neurons

To confirm whether we could differentiate iPSCs into spinal motor neurons in our culture system, we differentiated iPSCs derived from a control individual (wild-type [WT] iPSCs; 201B7 line [25, 26]) into spinal motor neurons using a previously reported protocol with modification [17, 21–23] (Fig. 3A). The protocol uses the SFEbQ method and stepwise treatment using the combination of dorsomorphin, SB, RA, PMN, BDNF, GDNF, AA, and CAMP. To confirm spinal motor neuron induction, we determined the expression of TUJ1 and H89, which are early motor neural cell markers. We also determined the expression of SMI-32 and CHAT, which are mature motor neural cell markers, using
immunostaining (Fig. 3B). Most of the induced spinal motor neurons expressed TUJ1 or SMI-32, although GFAP-positive astrocytes were also found around them (Fig. 3B). Most of the CHAT-positive motor neurons coexpressed HB9, indicating these cells were spinal motor neurons. In addition, RT-PCR analysis revealed that the expression levels were increased for PAX6, OIG2, HB9, and CHAT (motor neuron markers) and decreased for OCT3/4 (undifferentiated marker) (Fig. 3C). Taken together, these findings suggest that spinal motor neurons were induced from the iPSCs in our culture system and were useful for the analysis of SMA pathology.

Development of In Vitro Disease Model Using SMA-iPSCs

To establish a human in vitro disease model, we differentiated SMA-iPSCs to spinal motor neurons and evaluated their differences compared with those derived from WT-iPSCs. Immunostaining analysis showed that NESTIN, a marker specifically expressed in neural stem cells, was expressed in both differentiated SMA-iPSCs and WT-iPSCs for 14 days (Fig. 4A). In addition, RT-PCR analysis showed that expression of NESTIN was activated similarly in both differentiated SMA-iPSCs and WT-iPSCs for 14 days (data not shown). These findings suggest that both SMA-iPSCs and WT-iPSCs can be differentiated into NESTIN-positive neural stem cells within 14 days. However, when SMA-iPSCs were differentiated into spinal motor neurons, some differences were observed, including the dendrite and axon development of the iPSC-derived spinal motor neurons. The SMA-iPSC-derived spinal motor neurons showed a significantly reduced TUJ1 area that indicated shorter dendrite and axon length and impaired branching compared with the WT-iPSC-derived spinal motor neurons (WT-iPSCs, 30.2 ± 2.22 × 10^6 μm²; SMA-iPSCs, 16.4 ± 2.39 × 10^6 μm²; Fig. 4B, 4C). In addition, GFAP-positive astrocytes were activated in the differentiated SMA-iPSCs but were decreased in the differentiated WT-iPSCs (WT-iPSCs, 5.22% ± 0.41%; SMA-iPSCs, 49.3% ± 13.6%; Fig. 4B, 4D). Furthermore, we investigated the rate of increase in cleaved caspase 3-positive cells using immunostaining and FACS analysis. The results showed increased apoptosis in SMA-iPSC-derived spinal motor neurons (WT-iPSCs, 3.57% ± 1.44%; SMA-iPSCs, 27.8% ± 1.76%; Fig. 4B, 4E), resulting in a decreased rate of CHAT and HB9 double-expressed cells in differentiated SMA-iPSCs (Fig. 4B, 4F; supplemental online Fig. 1). In our culture system, approximately 40%–50% HB9-positive cells were observed in differentiated WT-iPSCs 56 days after motor neuron induction and approximately 10%–15% HB9-positive cells in differentiated SMA-iPSCs (WT-iPSCs, 46.0% ± 11.9%; SMA-iPSCs, 9.69% ± 4.83%; Fig. 4B, 4F). These phenotypic differences have been previously reported in vitro and in vivo, and, notably, astrocyte activation might contribute to motor neuron loss.

Next, to confirm the clone-to-clone variability of SMA-iPSC lines from our patient, we differentiated three SMA-iPSC lines into spinal motor neurons in our culture system. We found that the efficiency of spinal motor neuron differentiation and phenotypes of the spinal motor neurons showed little difference among these three SMA-iPSC lines (supplemental online Fig. 2). These results revealed that these phenotypic differences are related to SMA pathology rather than clone-to-clone variability.

Together, we were able to establish an in vitro disease model using phenotypic differences of the spinal motor neurons derived from both SMA-iPSCs and WT-iPSCs. In addition, this human in vitro SMA-iPSC model reflects SMA-specific pathology.

Identification of Efficacy of TRH Analog Using a Human SMA Model

To confirm that the TRH analog treatment can rescue SMA pathology, we treated SMA-iPSC-derived spinal motor neurons with the TRH analog using human in vitro SMA-iPSC model. Initially, we investigated whether TRHR1 was expressed in SMA-iPSC-derived spinal motor neurons. We found that the SMA-iPSC-derived spinal motor neurons and SMA-FbCs, but not the SMA-iPSCs, were positively stained with the TRHR1 antibody (Fig. 5A).

Next, we studied whether the TRH analog treatment could increase SMN protein expression in the cytoplasm and nuclear aggregate structures called gems. We found increased levels of SMN protein in SMA-iPSC-derived spinal motor neurons.
neurons and SMA-FbCs treated with the TRH analog (Fig. 5B; supplemental online Fig. 3A). Although the TRH analog treatment revealed efficacy at a modest concentration, we determined that 1 μM was the most effective concentration (supplemental online Fig. 3B). To further examine the change in the expression of SMN protein in the spinal motor neurons after the TRH analog treatment, we investigated the protein levels using Western blot analysis. We found that the protein level of SMN in differentiated SMA-iPSCs was restored after treatment with the TRH analog from 42 to 56 days of our induction protocol compared with the untreated group (WT-FbCs, 1.96 ± 0.48; no-treatment group, 0.27 ± 0.01; TRH analog-treated group, 0.35 ± 0.01; Fig. 5C, 5D). The data in Figure 5D are presented as the mean ± SEM.

Figure 2. Proof of iPSCs derived from SMA patient. (A): Newly generated iPSCs fully reprogrammed from fibroblasts of a SMA patient. Scale bars = 200 μm. (B): No karyotypic abnormalities were found in SMA-iPSCs used in the present study. (C): Reverse transcription-polymerase chain reaction indicating SMA-iPSCs and SMA-FbCs showed decreased SMN1 gene transcripts after Dde1 treatment. (D): ALP enzymatic activities in SMA-iPSCs. Scale bars = 200 μm. Expression of pluripotent markers, including OCT3/4, NANOG, SOX2, SSEA4, TRA-1-60, and TRA-1-81. Scale bars = 100 μm. (E): Expression of ectoderm, endoderm, and mesoderm markers in SMA-iPSCs by embryonic body formation. Scale bars = 100 μm. Abbreviations: Δ7-SMN, SMN2 lacking exon 7; ALP, alkaline phosphatase; FbCs, fibroblast cells; FL-SMN, full-length survival motor neuron gene; iPSCs, induced pluripotent cells; NANO, homeobox transcription factor nanog; OCT3/4, octamer-binding transcription factor 3/4; SMA, spinal muscular atrophy; SMN, survival motor neuron; SOX2, sry-box 2; SOX17, sry-box 17; SSEA1, stage-specific embryonic antigen 1; SSEA4, stage-specific embryonic antigen 4; TRA-1-60, tumor-related antigen-1-60; TRA-1-81, tumor-related antigen-1-81; WT, wild type (control individual).
Mechanisms of the Efficacy of the TRH Analog

To identify the mechanism of the TRH analog-induced SMN protein increase, we initially performed qPCR analysis of FL-SMN and Δ7-SMN mRNA. Using an RNA sample of differentiated spinal motor neurons at day 56, we also quantified the relative expression levels of FL-SMN and Δ7-SMN mRNA. The results showed an increased expression level of both FL-SMN (WT-FbCs, 1; no-treatment group, 0.04 ± 0.02; TRH analog-treated group, 0.35 ± 0.07) and Δ7-SMN (WT-FbCs, 1; no-treatment group, 5.47 ± 1.20; TRH analog-treated group, 7.42 ± 0.55) mRNA after TRH analog treatment in differentiated SMA-iPSCs (Fig. 6A, 6B). Therefore, we confirmed that the TRH analog promoted the transcriptional activation of SMN2 and increased the amount of SMN protein levels in spinal motor neurons.

Moreover, the TRH analog was previously reported to cause decreases of 75% in GSK-3β and 90% in Tau phosphorylation in cultured rat hippocampal neurons [27]. In addition, Makhortova et al. recently reported that inhibitors of GSK-3 could elevate SMN levels by stabilizing the SMN protein [28]. We examined whether GSK-3β inhibition was caused by the TRH analog treatment in spinal motor neurons derived from SMA-iPSCs, and Western blot analysis showed that the TRH analog treatment inhibited GSK-3β activity (Fig. 6C). Although our Western blot analysis results...
revealed an increase in the band of phosphorylated GSK-3β after the TRH analog treatment compared with the untreated group, it is known that phosphorylation of GSK-3β indicates inhibition of enzyme activity [29]. Therefore, these results revealed that SMN protein levels can be increased, at least through transcriptional activation of the SMN2 gene and inhibition of GSK-3β activity in these SMA-iPSCs.

Dendrite and Axon Development Efficacy of the TRH Analog

Recently, the SMN protein was reported to be required for neuronal differentiation, particularly for dendrite and axon development [30]. Therefore, we compared the efficacies of the TRH analog and VPA in enhancing dendrite and axon development.
VPA is a histone deacetylase inhibitor, which increases SMN protein levels in SMA patients through activation of SMN2 transcription [12]. Therefore, its therapeutic effects are expected to be similar to those of the TRH analog. The dendrite and axon development action of the TRH analog or VPA in SMA-iPSC-derived spinal motor neurons was determined by evaluating the existence of the region of TUJ1-positive cells. The presence of TUJ1-positive cells was determined in the WT-iPSC-derived and untreated SMA-iPSC-derived spinal motor neurons and in the groups treated with the TRH analog or VPA from 42 to 56 days of our induction protocol. It could be seen in the typical example that the spinal motor neurons derived from the WT-iPSCs were thicker, longer, and more morphologically mature than were the SMA-iPSCs (Fig. 6D; supplemental online Fig. 4). The density of neuronal dendrite and axon in the differentiated WT-iPSCs was also higher compared with the differentiated SMA-iPSCs (Fig. 6D, 6E). The TRH analog- or VPA-treated groups significantly improved the phenotype of neuronal dendrite and axon in the differentiated SMA-iPSCs, showing a phenotype closer to differentiated WT-iPSCs (WT-FbCs, 27.3 ± 3.81 × 10^6 μm²; no-treatment group, 6.45 ± 1.81 × 10^6 μm²; TRH analog-treated group, 13.1 ± 0.79 × 10^6 μm²; VPA-treated group, 14.7 ± 0.22 × 10^6 μm²; Fig. 6D, 6E; supplemental online Fig. 4). Therefore, the TRH analog or VPA significantly increased the existing region of the TUJ1-positive cells, suggesting that they have an effect on dendrite and axon development of spinal motor neurons derived from SMA-iPSCs. In addition, no significant differences were found between the efficacies of the TRH analog and VPA.

Finally, we studied the possible restorative effects against the activated GFAP-positive astrocytes and increased cleaved caspase 3-positive cells observed in the human in vitro...
SMA-iPSC model. However, these parameters were unchanged after treatment with the TRH analog or VPA (supplemental online Fig. 5).

Taken together, the TRH analog increased the SMN protein via transcriptional activation of the \( SMN2 \) gene and stabilized the SMN protein by inhibition of GSK-3\( \beta \) activity. In addition, the TRH analog had an effect on the dendrite and axon development of the spinal motor neurons of the SMA patients.

**DISCUSSION**

In the present study, we established a human in vitro SMA-iPSC model. Furthermore, we used this model to reveal aspects of SMA pathology, including loss of SMN protein expression (Fig. 4B), a short dendrite and axon length of spinal motor neurons (Fig. 4B, 4C), activated astrocytes (Fig. 4B, 4D), and an increase in the level of apoptosis (Fig. 4B, 4E, 4F). Numerous recent reports on human in vitro disease models from SMA-iPSCs have been
published [22, 23, 31–33]. Some drugs, compounds, and antibodies, including VPA [22], tobramycin [22], a Fas-blocking antibody [23], a caspase-3 inhibitor [23], N-acetylcysteine [31], and SMN2 splicing modifiers [33], have been analyzed using these models. However, no reports have been published on the efficacy and mechanism of action of TRH analog treatment of SMA in a human in vitro SMA-iPSC model. In addition, no similar reports have been published involving SMA-FbCs. To the best of our knowledge, we report the first study to show that a human in vitro SMA-iPSC model can be used to analyze the effects of the TRH analog treatment.

TRH is a peptide hormone that is secreted by the hypothalamus and releases prolactin and thyrotropin from the pituitary gland; it also plays a critical role in metabolic regulation [34]. As before, a number of reports have indicated that the TRH analog is effective in neurodegenerative diseases. The administration of the TRH analog in experimental rats promoted the release of acetylcholine in the cerebral cortex and hippocampus [35] and increased the cerebral blood circulation volume [36]. Also, the TRH analog protects neurons in the embryonic stage against glutamine toxicity; this protective effect is concentration dependent [37]. As reported in the study involving spinal motor neurons and the TRH analog, administering the TRH analog, 3-methyl-(-)-5,6-dihydroxytyl-L-histidyl-L-prolinamide, to spinal ventral horn neurons taken from rat embryos significantly elongated the axons [38]. In addition, it was reported that treatment with a metabolite of TRH, cyclo-(His-Pro), suppresses streptozotocin-induced apoptotic events such as activation of caspase 3 [39]. The TRH analog exerts neuron stimulating and protective effects. TRH analog treatment has been used in patients with persistent disturbances in consciousness or spinocerebellar degeneration in Japan. We have also investigated TRH analog treatment for SMA patients in clinical trials. However, the mechanisms of the neuron-stimulating and neuron-protective effects of the TRH analog remain unidentified.

TRH and its analog bind to a specific receptor, which exists as 2 isotypes, namely TRHR1 and TRHR2. These receptors belong to the rhodopsin/β-adrenalin receptor-like G protein-coupled family, and humans express only TRHR1 [40]. TRHR1 expression is widely distributed in the cortex, thalamus, hypothalamus, midbrain, hindbrain, cerebellum, amygdala, septal area, hippocampus, pituitary gland, and α-motor neuron in the spinal cord. After binding to the receptor, the TRH analog is thought to act through some signal transduction mechanisms [40]. Recent reports have described the neuroprotective actions of the TRH analog as being mediated via inhibition of GSK-3β [27]. In addition, inhibition of GSK-3β was reported to increase SMN protein levels by blocking its degradation system [28]. In particular, Rubin et al. performed a cell-based screen and identified that platelet-derived growth factor (PDGF) produced the greatest increase in SMN protein levels [28]. PDGF increased SMN protein, in part, by inhibiting GSK-3β, but motor neurons lack PDGF receptors [28]. In the present study, we first confirmed that TRHR1 is expressed in spinal motor neuron-derived SMA-iPSCs as SMA-FbCs (Fig. 5A, 5B). In addition, we found that treatment with the TRH analog led to GSK-3β inhibition and increased the level of SMN protein in spinal motor neuron-derived SMA-iPSCs, using Western blot analysis (Fig. 6C), involving activation of the transcription of the SMN2 gene using RT-PCR. In the present study, we elucidated an aspect of the mechanism of the TRH analog action against our patient with SMA.

Many developed treatments have been fraught with problems that have limited their effectiveness in clinical trials. VPA has been reported to raise the protein levels of SMN in the human in vitro SMA-iPSC model [22]. However, clinical trials have not demonstrated the benefits of VPA treatment in SMA patients [41]. Similarly, the TRH analog was not effective in all SMA patients in our clinical trials. The inconsistencies in the data from in vitro and clinical studies might be explained by the coexistence of responders and nonresponders to the drugs. We postulated that an established human in vitro SMA-iPSC model could serve as a preselection assay system for potential responders and, in particular, can be used to analyze the differences in genetic background, including SMN and neuronal apoptosis inhibitory protein (NAIP) gene deletion, SMN gene deletion only, and no SMN and NAIP gene deletion. In addition, we can analyze the combinational effects of different therapeutic agents on the mechanisms involved in the human in vitro SMA-iPSC model in future.

**Conclusion**

We have established a human in vitro SMA-iPSC model and elucidated an aspect of the mechanisms involved in the efficacy of the TRH analog in treating SMA pathology. Consequently, we will be able to assay a novel therapeutic drug or drug combination for the treatment of SMA using this human in vitro SMA-iPSC model.

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**Author Contributions**

K.O.: collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; M.F.: conception and design, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; Z.K.: conception and design, provision of study material or patients, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; J.S. and C.K.: collection and/or assembly of data, final approval of manuscript; Y.T.: data analysis and interpretation; Y.O., Y. Nagahara, Y. Noda, T.K., S.A., and K.T.: provision of study material or patients, collection and/or assembly of data, final approval of manuscript; M.S., H.H., and H.K.: conception and design, final approval of manuscript.

**Disclosure of Potential Conflicts of Interest**

The authors indicated no potential conflicts of interest.
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