Title: Abnormal mitochondrial transport and morphology as early pathological changes in human models of spinal muscular atrophy

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SUMMARY STATEMENT

This study provides the first evidence of impaired mitochondrial dynamics in human models of spinal muscular atrophy, which serve as potential therapeutic targets for this devastating disease.
Spinal muscular atrophy (SMA), characterized by specific degeneration of spinal motor neurons, is caused by mutations in the survival motor neuron 1 (SMN1) gene and subsequent decreased levels of functional SMN. How the deficiency of SMN, a ubiquitously expressed protein, leads to spinal motor neuron-specific degeneration in SMA patients remains unknown. In this study, we examined the role of SMN on mitochondrial axonal transport and morphology in human motor neurons by generating SMA type 1 patient-specific induced pluripotent stem cells (iPSCs) and then differentiating these cells into spinal motor neurons. The initial specification of spinal motor neurons was not affected, but these SMA spinal motor neurons specifically degenerated following long-term culture. Moreover, at an early stage in SMA spinal motor neurons, but not in SMA forebrain neurons, mitochondrial number, area, and transport were significantly reduced in axons. Knocking down of SMN expression led to similar mitochondrial defects in spinal motor neurons derived from human embryonic stem cells (hESCs), confirming that SMN deficiency results in impaired mitochondrial dynamics. Finally, the application of N-acetylcysteine (NAC) mitigated the impaired mitochondrial transport and morphology, and then rescued motor neuron degeneration in SMA long-term cultures. Furthermore, NAC ameliorated the reduced mitochondrial membrane potential in SMA spinal motor neurons, suggesting that NAC may rescue apoptosis and motor neuron degeneration by improving mitochondrial health. Together, our data demonstrate that SMN deficiency results in abnormal mitochondrial transport and morphology and subsequent reduced mitochondrial health, which are implicated in the specific degeneration of spinal motor neurons in SMA.
INTRODUCTION

Spinal muscular atrophy (SMA), the leading genetic cause of death in infants and toddlers, is characterized by spinal motor neuron-specific degeneration and subsequent muscles weakness and paralysis (Pearn, 1978; Pearn, 1980). This devastating disease is caused by homologous deletion or mutations of the telomeric copy of the survival motor neuron 1 (SMN1) gene, leading to decreased levels of functional SMN proteins (Burglen et al., 1996; Lefebvre et al., 1995). Though SMN protein is ubiquitously distributed, reduction of functional SMN has a profound effect on spinal motor neurons, leading to specific degeneration of these cells in SMA patients. The prominent pathophysiological changes in SMA are axonal and neuromuscular junction abnormalities (Jablonka et al., 2004; Kong et al., 2009; McWhorter et al., 2003; Rossoll et al., 2003). How axonal and synaptic functions are affected and why motor neurons specifically degenerate in SMA remain largely unclear.

SMN protein is concentrated in discrete foci called gems in the nucleus of many cell types, which has a housekeeping role in mRNA splicing (Akten et al., 2011; Zhang et al., 2006). In addition to their nuclear locations, SMN proteins are also present in the dendrites and axons of neurons and are associated with microtubules (Fan and Simard, 2002; Zhang et al., 2003). SMN is important for the assembly of axonal messenger ribonucleoprotein complexes (mRNPs) and can interact with a variety of mRNA binding proteins (mRNPs) in neurons (Akten et al., 2011; Fallini et al., 2014; Kanai et al., 2004; Liu and Dreyfuss, 1996; Rossoll et al., 2002), which may affect axonal transport or the local translation of mRNA at synapses.

A recent study reported the dysfunction of mitochondria in mouse NSC-34 cells whose SMN expression was knocked down using siRNA, suggesting that SMN is important for mitochondrial function (Acsadi et al., 2009). The depletion of mitochondrial DNA (Berger et al., 2003; Ripolone et al., 2015) and increased oxidative stress (Hayashi et al., 2002) have also been reported in SMA patients. Our previous study (Wang et al., 2013) showed that the
production of mitochondrial superoxide is significantly increased in spinal motor neurons, but not in forebrain neurons, derived from SMN-knockdown human embryonic stem cells (hESCs). Considering that spinal motor neurons are large cells that have a high energy demand, impaired mitochondrial function may be involved in the axonal defects and specific motor neuron degeneration in SMA. Spinal motor neurons have long axons and the axonal transport of mitochondria is important for the synthesis of ATP in areas of axoplasm distant from the cell body. Reduced mitochondrial axonal transport has been observed in spinal motor neurons isolated from mouse models of amyotrophic lateral sclerosis (ALS) (De Vos et al., 2007; Magrane et al., 2014), a motor neuron disease characterized by axonal degeneration. Interestingly, the defects of retrograde mitochondrial axonal transport were observed at an early stage before the onset of the symptoms (Magrane et al., 2014), implying the abnormal mitochondrial transport in axonal degeneration of spinal motor neurons in ALS. Similar as ALS, axonal degeneration is a common pathology in SMA, but the age of onset of SMA is much earlier. Whether the transport and morphology of mitochondria are impaired in SMA motor neurons and whether they play any role in motor neuron degeneration in SMA remain unknown.

Human pluripotent stem cells (Takahashi et al., 2007; Thomson et al., 1998; Yu et al., 2007), which have the capacity to generate all types of cells in our body including spinal motor neurons, provide a unique source to researchers for studying the specific cell types that are affected by various diseases in vitro. Human models of SMA have been established by generating induced pluripotent stem cells (iPSCs) from SMA patients (Chang et al., 2011; Corti et al., 2012; Ebert et al., 2009; Sareen et al., 2012) and knocking down the functional SMN in hESCs (Wang et al., 2013), which recapitulate disease-specific degeneration in motor neurons. Here, using both iPSC- and hESC-based SMA models, we examined the mitochondrial axonal transport and morphology in these stem cell-derived neurons. Our data
reveal a significant reduction of mitochondrial transport, number, and size in axons of SMA spinal motor neurons, but not forebrain neurons, at an early stage before motor neurons are degenerated. Motor neurons derived from SMN-knockdown hESCs exhibit similar abnormal mitochondrial dynamics as those from SMA iPSCs, confirming the direct link between these mitochondrial defects and SMN deficiency. Moreover, application of NAC, which ameliorates the mitochondrial defects, also rescues the specific motor neuron degeneration, suggesting that mitochondrial defects underlie the motor neuron-specific degeneration in human SMA models.

RESULTS

Characterization and neural differentiation of control and SMA iPSC lines

We obtained fibroblast cells of SMA type 1 patients (Coriell Cell Repositories) and successfully generated iPSC clones using the episomal method (Okita et al., 2011). Control iPSC lines (wild type, WT) were also generated from fibroblast cells of a normal individual. The episomal vectors containing pluripotent factors are progressively lost from cells, leading to the generation of iPSCs free of vector and exogenous sequence. We then analyzed iPSC lines that were derived from both the SMA type1 and WT fibroblast cells. These iPSC lines exhibited characteristic hESC-like morphology and expressed the pluripotency markers NANOG, TRA-1-60, and SSEA4 (Fig.1A). To further validate the pluripotency of the iPSC lines, we examined the formation of teratomas after injecting the iPSCs into SCID mice. Both the SMA type1 and WT iPSC lines were pluripotent as revealed by their ability to spontaneously differentiate into tissues of each of the three germ layers (Fig. 1B). Considering that iPSCs are susceptible to chromosomal abnormalities, we then performed
karyotype analysis. As shown in Figure 1C, normal karyotyping was maintained even after multiple passages.

To successfully model the disease, another important criterion is the maintenance of gene mutations during reprogramming and subsequent differentiation. In SMA patient iPSCs, homologous deletion of the \( SMN1 \) gene results in reduced levels of functional SMN. As expected, the mRNA expression of functional SMN (SMN-full length, SMN-FL) was significantly decreased in SMA type1 iPSC lines compared with the WT (Fig. 1D). Using a primer set that is specific to the \( SMN1 \) gene, we then examined the expression of \( SMN1 \) gene in DNAs isolated from control and SMA iPSCs. RT-PCR analysis showed that SMA iPSCs were absent of \( SMN1 \) gene, confirming the loss of \( SMN1 \) gene (Fig. 1E). At the protein level, the expression of SMN-FL protein in SMA Type1 iPSCs was significantly decreased compared to that in WT iPSCs (~20% of the control, Fig. 1F), confirming reduced level of functional SMN in SMA iPSCs.

In order to compare the spinal motor neurons from control and SMA iPSCs, we differentiated these iPSCs into spinal motor neurons using a differentiation protocol modified from our previous methods (Li et al., 2005; Li et al., 2008; Zeng et al., 2010). Human iPSCs were first differentiated to neuroepithelial cells, which were then treated with retinoic acid (RA) for caudalization and purmorphamine for ventralization (Chen et al., 2014; Du et al., 2015) (Fig. 2A). Motor neuron-enriched progenitors were isolated and suspended at 2 weeks after differentiation from iPSCs. For terminal differentiation, motor neuron-enriched clusters were dissociated and plated onto polyornithine/laminin-coated coverslips at day 19. Five days after plating, these coverslips were fixed and subjected to HB9 (a marker for spinal motor neuron) and Tau (an axonal marker) immunostaining (Fig. 2B). The proportion of HB9\(^+\) postmitotic motor neurons was around 60% and there were no significant differences between control and SMA groups (Fig. 2C). These data suggest that spinal motor neurons can be
efficiently specified from SMA iPSCs and the initial specification of HB9+ spinal motor neurons from SMA iPSCs is not altered.

**SMA iPSC-derived spinal motor neurons exhibited reduced mitochondrial axonal transport**

SMA is characterized by axonal and synaptic defects and recent studies reported the dysfunction of mitochondria in SMA cell models. To understand the mechanisms underlying the functional defects of spinal motor neurons in SMA, we assessed the mitochondrial transport in SMA type1 iPSC derived spinal motor neuron cultures. Using the MitoTracker CMXRos dye, we first analyzed mitochondrial axonal transport in day 24 iPSC derived neurons with live-cell imaging. As shown in Figure 3A, representative kymographs revealed the nature of axonal transport in the neuron. The frequency of motile events was calculated by counting the number of times each mitochondrion moved with a velocity of >300nm/s. This velocity threshold was selected to exclude actin-mediated transport events which fall well below this threshold (De Vos and Sheetz, 2007). Calculation of the percentage of motile mitochondria for each cell revealed a significant reduction in SMA Type1 iPSC-derived spinal motor neuron cultures as compared with the WT (Fig. 3B), and the frequency of motile events was also reduced in the SMA neurons (Fig. 3C).

Transport vesicles and membranous organelles can move in two different directions, i.e., from cell bodies down to the axon (anterograde transport) and from distal part back to the cell body (retrograde transport). It is unknown whether anterograde or retrograde transport is affected in SMA. Therefore, using these SMA patient iPSC-derived spinal motor neuron cultures, we further analyzed the mitochondrial transport in both anterograde and retrograde directions. Comparison of mitochondrial transport velocities in both retrograde (Fig. 3D) and anterograde (Fig. 3E) directions showed that there were no significant differences between
control and SMA neurons. There was a significant reduction in the frequency of motile events in the SMA Type1 iPSC derived spinal motor neuron cultures as compared to WT. Further analysis of the direction of transport revealed a non-significant trend toward the reduction of events in the anterograde direction (Fig. 3G) and a significant reduction in retrograde events in SMA cells (Fig. 3F). These results suggest that mitochondrial axonal transport is impaired in SMA motor neurons at an early stage during the disease progression, which may serve as an early contributor to motor neuron degeneration in SMA.

**N-acetylcysteine rescued the mitochondrial transport and morphology defects**

Our previous study showed that NAC, an antioxidant, could mitigate the increased mitochondrial oxidative stress caused by knocking down of SMN, leading to the rescue of motor neuron degeneration (Wang et al., 2013). To further dissect the protective role of NAC in SMA, we examined the effect of NAC on the mitochondrial axonal transport in SMA spinal motor neuron cultures. NAC (80 μg/ml) was added to neural cultures derived from SMA iPSCs from the neural progenitor stage to motor neuron stage (day 13 to day 24) and mitochondrial axonal transport was examined at day 24 as described above. As shown in Figure 3, application of NAC significantly increased the percentage of motile mitochondria (Fig. 3B) compared to that in SMA motor neuron cultures. These data suggests that NAC can ameliorate the mitochondrial axonal transport defects in SMA motor neurons.

During the investigation of mitochondrial axonal transport in vitro, we constantly observed smaller mitochondria in SMA spinal motor neuron cultures compared to controls. Since mitochondrial dynamics and distribution are important for their functions, we further analyzed the mitochondrial number and morphology in axons of spinal motor neuron cultures at day 24. As shown in representative images of mitochondrial morphology (Fig. 4A), the number of mitochondria in SMA Type1 iPSC-derived spinal motor neuron culture was
significantly decreased compared to that in WT group (Fig. 4B). Though the average length of mitochondria was non-significantly decreased in SMA Type1 iPSC-derived spinal motor neuron cultures (Fig. 4C), the mitochondrial area was significantly decreased compared with WT (Fig. 4D). After the treatment of NAC, the mitochondrial number was significantly increased compared to that in SMA motor neuron cultures (Fig. 4B). Together, our data suggest that in SMA spinal motor neurons, there are deficits in mitochondrial axonal transport, distribution, and morphology, which can be partially rescued by the application of NAC.

**Knocking down SMN-full length in spinal motor neurons resulted in similar mitochondrial transport and morphology deficits**

To confirm whether the abnormal mitochondrial dynamics we observed in SMA spinal motor neuron cultures are directly linked to the deficiency of SMN, we examined the transport and morphology of mitochondria in spinal motor neurons derived from SMN-knockdown hESCs. In our previous study, we have established SMN-FL knockdown hESC lines which recapitulate the pathological changes in SMA (Wang et al., 2013). Using the SMN-FL RNAi and luciferase RNAi (as a control) hESCs, we then differentiated these stem cells into neural lineage and spinal motor neuron as we described for SMA iPSCs. In day 24 neurons, mitochondrial axonal transport was examined after staining with MitoTracker (Fig. S1A-G). Similarly as observed in SMA iPSC-derived cultures, we observed significant reductions in the percentage of motile mitochondria (Fig. S1B) and the frequency of motile events at the retrograde direction (Fig. S1F) in the SMN-FL knockdown spinal motor neuron cultures as compared with control luciferase RNAi cultures. The frequency of motile events had a trend to decrease but was not statistically significant in the SMN-FL knockdown neurons (Fig. S1C). Together, the SMN-knockdown spinal motor neuron cultures showed similar reductions in the motile mitochondria and the frequency of motile events at the retrograde
direction, confirming the direct link between SMN deficiency and the mitochondrial transport
deficits.

Next, we compared the mitochondrial number and morphology in axons between luciferase and SMN-FL RNAi spinal motor neuron cultures (Fig. S2). As shown in representative images of mitochondrial morphology (Fig. S2A), the number of mitochondria in SMN-FL RNAi hESC-derived spinal motor neuron cultures was significantly decreased compared to that in luciferase RNAi group (Fig. S2B). Similarly as in SMA iPSC-derived motor neuron cultures, the mitochondrial area was significantly decreased in SMN-knockdown spinal motor neurons (Fig. S2D). These data confirm that loss of SMN-FL function directly implicated in the abnormal mitochondrial transport, distribution, and size in SMA spinal motor neurons. Furthermore, the application of NAC to the SMN-knockdown cultures (day 13 to day 24) significantly mitigated the reduction of the percentage of motile mitochondria (Fig. S1B) and the number and area of mitochondria in axons (Fig. S2 B, D), confirming the protective effects of NAC against mitochondrial defects in both iPSC- and hESC-based SMA models.

**Cell type-specific alterations of mitochondrial axonal transport and morphology in SMA**

Considering that spinal motor neurons specifically degenerate in SMA patients, we then tested whether the mitochondrial defects are specific to spinal motor neurons and are not observed in other neuron types. To achieve this, we generated forebrain neurons (telencephalic glutamatergic neurons) from WT and SMA Type 1 iPSCs and examined the mitochondrial axonal transport and morphology in these neurons. The iPSC lines were differentiated to forebrain neurons using a paradigm we established previously, which leads to the efficient generation of telencephalic progenitors (FOXG1+) and subsequent TBRI+
glutamatergic neurons (Fig. 5A) (Boisvert et al., 2013; Li et al., 2009; Zeng et al., 2010). Forebrain neurons were dissociated and plated on coverslips for terminal differentiation. At the same time point as we tested for the motor neuron cultures (day 24), the mitochondrial transport and morphology were examined and compared between WT and SMA forebrain neurons (Fig. 5B). Interestingly, there were no significant alterations in the mitochondrial axonal transport (Fig.5 C-H) and mitochondrial morphology (Fig.5 I-K) in SMA forebrain neurons compared to that in WT neurons. Together, these data suggest that abnormal mitochondrial dynamics in our human SMA models are specific to spinal motor neurons, which may underlie the specific degeneration of spinal motor neurons in SMA.

Specific degeneration of spinal motor neurons in long-term cultures

A recent study reported that ALS iPSC-derived spinal motor neurons underwent degeneration and exhibited bead-like swellings along the neurites (Chen et al., 2014). Since the SMA iPSC-derived spinal motor neurons exhibited the impaired mitochondrial axonal transport, we asked if these neurons underwent axonal degeneration, exhibited bead-like swellings, and died in long-term culture. To test this, we cultured the spinal neurons on coverslips in the presence of neurotrophic factors for another 3 weeks (total 42 days after differentiation from iPSCs). Then, we performed HB9 and Tau staining to examine the formation of axonal swelling (Fig. 6A). Our data showed that the number of axonal swellings in the SMA spinal motor neurons was significantly increased compared to that in the WT spinal motor neurons (Fig. 6B). Next, in order to examine whether these neurons undergo apoptosis, we compared the caspase 3/7 activity between SMA and WT motor neuron cultures. In spinal motor neuron cultures (day 42), the activity of caspase 3/7 significantly increased in the SMA spinal motor neurons compared with that in the WT group (Fig. 6C). Interestingly, at the same time point (day 42) in forebrain neuron cultures, there were no significant differences in the number of axonal swellings (Fig. 6B) or the caspase 3/7 activities (Fig. 6C) between SMA Type1 and
WT groups. These results reveal that SMA iPSC-derived spinal motor neurons specifically degenerate in long-term cultures, recapitulating the selective vulnerability in SMA.

Next, we examined whether NAC, which mitigated the abnormal mitochondrial dynamics, was able to rescue the motor neuron degeneration in long-term cultures. NAC was added to SMA spinal motor neuron cultures during the same time period (from day 13 to day 42) and the formation of bead-like axonal swellings was analyzed at 42 days after differentiation (Fig. 6A). After NAC treatment, the number of axonal swellings was significantly decreased compared to SMA motor neuron cultures (Fig. 6B). Moreover, NAC ameliorated the increased caspase 3/7 activity in SMA motor neuron cultures (Fig. 6C), suggesting that NAC can rescue the specific motor neuron degeneration in long-term cultures in the SMA human model. To further confirm the protective effect of NAC, we established iPSC lines from a second SMA patient and examined the effect of NAC in SMA Patient 2 iPSC-derived motor neurons. The SMA Patient 2 iPSC-derived motor neurons exhibited similar phenotypes including increased axonal swellings (Fig. 7A,B) and increased apoptosis (Fig. 7C), which can also be rescued by NAC (Fig. 7A-C). Together, these data reveal that application of NAC, which ameliorates the mitochondrial defects, also rescues the specific motor neuron degeneration, suggesting that mitochondrial defects underlie the motor neuron-specific degeneration in human SMA models.

Considering that alterations in mitochondrial membrane potential can result in the release of cytochrome C and apoptosis (Gottlieb et al., 2003), we sought to examine whether mitochondrial health was affected in SMA derived motor neurons. The cells were incubated with the fluorescent dye TMRM, which binds to mitochondria based on the membrane potential (Perry et al., 2011). We compared the TMRM fluorescence intensity in motor neuron cultures derived from WT, SMA, and SMA plus NAC treated groups at around 5 weeks after differentiation (day 36, before motor neurons degenerated; Fig. 7D). This showed
a significant reduction in TMRM fluorescence in SMA iPSC-derived motor neuron cultures compared to WT (Fig. 7E), indicating reduced mitochondrial health. The TMRM signals were significantly inhibited in all groups by applying FCCP, a mitochondrial uncoupler (Benz and McLaughlin, 1983), confirming the specificity of TMRM labeling. After NAC treatment, the TMRM fluorescence was significantly increased compared to SMA motor neuron cultures (Fig. 7D,E), indicating the improvement of mitochondrial health by NAC. Together, these data suggest that mitochondrial dysfunction including reduced mitochondrial health in SMA spinal motor neurons is implicated in the pathogenesis of SMA.

**DISCUSSION**

Mitochondrial dysfunction has been implicated as a critical pathological abnormality in many neurodegenerative diseases, such as ALS (Magrane et al., 2014), hereditary spastic paraplegia (Denton et al., 2014), Parkinson’s disease, Alzheimer’s disease and Huntington’s disease (Chen and Chan, 2009). Studies have reported mitochondrial dysfunction and oxidative stress in SMA models and patients’ autopsy or biopsy samples (Acsadi et al., 2009; Berger et al., 2003; Hayashi et al., 2002; Ripolone et al., 2015; Wang et al., 2013). However, the relationship between abnormal mitochondrial function and the pathogenesis of SMA remains largely unclear. SMA is characterized by the axonal and synaptic defects in spinal motor neurons. In this study, we found that the transport and density of axonal mitochondria were significantly reduced in spinal motor neurons, but not in forebrain neurons. Considering that mitochondria are important for providing energy, impaired mitochondrial axonal transport and numbers in axons may be implicated in the axonal defects in SMA spinal motor neurons. Interestingly, these alterations in mitochondria happened at an early stage in motor neurons, within 1 week after the initial specification of spinal motor neurons. This suggests that SMA
spinal motor neurons exhibit early defects even the initial differentiation efficiency is not altered. This is also in line with previous findings that the axonal outgrowth and neurite complexity were impaired in SMA motor neurons before they degenerate (Chang et al., 2011; Wang et al., 2013). Interestingly, though the mitochondrial transport in both anterograde and retrograde directions has a trend in decrease, the retrograde transport is significantly decreased in both SMA iPSC- and SMN-knockdown motor neurons. Our finding agrees with a recent report on the mitochondrial transport using neurons isolated from ALS mouse models, where the retrograde transport in motor neuron axons was first affected before the onset of the symptoms (Magrane et al., 2014). The early impairment of retrograde mitochondrial transport implies its role in the motor neuron degeneration. Retrograde axonal transport is important for proper responses of neurons as it brings distal trophic factors or stress stimuli to the soma. The role of retrograde axonal transport deficits in neurodegeneration is further supported by studies showing that mutations in subunits of cytoplasmic dynein, a motor protein involved in retrograde transport, can result in motor neuron degeneration (Hafezparast et al., 2003; Puls et al., 2003). Whether the impaired retrograde transport is specific to mitochondria or whether retrograde transport in general is affected in our SMA models needs to be further investigated.

Mitochondria undergo continuous fission and fusion to maintain normal shape and function (Chan, 2012; van der Bliek et al., 2013). Analysis of the mitochondrial morphology in SMA motor neurons reveal a significant reduction of mitochondrial density (or number) and area along axons. Alterations in mitochondrial morphology/dynamics have been previously reported in other motor neuron diseases (Magrane et al., 2014) but not in SMA. Our study provides new evidence on the involvement of abnormal mitochondrial dynamics in SMA motor neurons. Similarly as the impaired mitochondrial axonal transport, the reduced mitochondrial density and area were also observed in motor neurons at an early stage (day 24
neurons). The reduced mitochondrial area in SMA spinal motor neurons is probably caused by the reduction of both length and width which all showed a trend of reduction. Though it is still not clear why the density and size of mitochondria are reduced in SMA spinal motor neurons, mitochondrial size can be affected by fission/fusion (Chan, 2012; van der Bliek et al., 2013). As shown from previous studies, increased mitochondrial fission could result in reduced mitochondrial size, leading to apoptosis (Itoh et al., 2013; Nakamura and Lipton, 2010). To examine mitochondrial health in SMA motor neuron cultures, we measured mitochondrial membrane potential using the fluorescence dye TMRM (Perry et al., 2011). Our data showed a significant reduction of TMRM fluorescent in SMA derived spinal motor neuron cultures at around 5-weeks (day 36), indicating reduced mitochondrial health. Interestingly, this reduction was not observed in SMA cultures at early stages (day 24) when mitochondrial dynamics were impaired. Consideration that alterations in mitochondrial membrane potential can result in the release of cytochrome C and apoptosis (Gottlieb et al., 2003), it is possible that impaired mitochondrial dynamics may cause reduced mitochondrial health, leading to the degeneration of spinal motor neurons in SMA. In the future, it would be interesting to further dissect how mitochondrial dynamics and health are affected at different stages of disease progression and how these mitochondrial related changes (transport, morphology and health) interact with each other.

SMA is caused by decreased levels of functional SMN which is a ubiquitously expressed protein. How the decreased level of SMN leads to the mitochondrial dysfunction in SMA neurons is not clear. Nuclear SMN plays an important role in the assembly of many different classes of small ribonucleoprotein particles (snRNPs) that function in pre-mRNA splicing and gene transcription. Recently, a study reported that SMN-dependent U12 splicing events are impaired in SMA models, leading to decreased expression of a subset of transcripts that utilize U12 splicing (Lotti et al., 2012). This brings up one possibility that the impaired
mitochondrial function observed in SMA may be a direct consequence of the loss of SMN’s housekeeping role in snRNP biogenesis and pre-mRNA splicing. The other possibility lies in the SMN’s role in mRNA transport in motor neuron axons. Impairment in transporting mRNAs that are important for mitochondria and motor neurons may result in mitochondrial dysfunction, leading to motor neuron degeneration. Although the detailed mechanisms are not clear, the early alterations in mitochondrial transport and morphology suggest that mitochondria can be a potential therapeutic target for SMA. Indeed, application of NAC, an antioxidant which was previously shown to reduce the mitochondrial oxidant stress, was able to ameliorate the mitochondrial transport and morphology alterations, improve the mitochondrial health, and rescue the subsequent motor neuron degeneration. The protective effects of NAC were observed in motor neuron cultures derived from two different SMA patient iPSCs, as well as SMN-knockdown hESCs, confirming the beneficial effects of NAC in human SMA cell models in vitro. By administering NAC in wobbler mice (Henderson et al., 1996) and animal models of ALS (Andreassen et al., 2000), NAC has shown some beneficial effects on reducing motor neuron degeneration in vivo. Whether NAC or improving mitochondrial function has beneficial effects on SMA mice in vivo and what is the time window for intervention requires further investigation.

Selective degeneration of certain types of human neurons is fundamental to many neurodegenerative diseases, but the underlying mechanisms are not known. The development of human pluripotent stem cells, which can differentiate into various neuronal subtypes, provides a unique system to study this fundamental question. Using human pluripotent stem cell-based models of SMA, in this study, we reveal that mitochondrial deficits including mitochondrial transport, distribution, and morphology are early pathological changes in human SMA models, which are implicated in the motor neuron-specific degeneration in SMA patients. How SMN deficiency in SMA results in mitochondrial dysfunction in spinal
motor neurons specifically needs to be further investigated. Better understanding of how and why mitochondrial dynamics and function are altered in SMA spinal motor neurons will provide valuable insights into identifying potential therapeutic targets for rescuing motor neuron degeneration in SMA.
MATERIALS AND METHODS

Reprogramming human fibroblasts into iPSC lines

Human iPSC lines were established from human fibroblasts by transfecting them with episomal plasmids (Addgene), as reported previously (Okita et al., 2011). Briefly, human fibroblasts obtained from SMA type 1 patients and normal individual (Coriell Cell Repositories) were seeded at ~10^5 cells/35-mm dish in DMEM supplemented with 10% fetal bovine serum (FBS) and 0.1 mM non-essential amino acids. For episomal transduction, human fibroblasts (~500,000) were dissociated and then infected with episomal plasmids containing pluripotency factors (Oct3/4, Sox2, L-Myc, Klf4 and Lin28). At round 1 week after electroporation transduction, cells were plated onto a 35-mm dish in DMEM supplemented with 10% FBS. After culturing for 7 days, cells were dissociated and seeded onto mouse embryonic fibroblast (MEF) feeder at ~10^5 cells/100-mm dish. Two weeks later, colonies with morphologies similar to hESCs were observed. These colonies were split onto MEF feeder cells derive iPSC lines. After several passages, homogenous colonies with ESC-like morphology were generated. The episomal iPSCs used in this study were WT (derived from GM05659, Coriell Cell Repositories) and two SMA Type1 (from GM03813 and GM00232, Coriell Cell Repositories) lines.

For testing the formation of teratoma, around 4 wells of 6-well plate of iPSCs were collected, dissociated, and resuspended in 50 μl hESC medium. These cells were injected using 1 cc U-100 Insulin Syringe into the hind limb of SCID mice (male, 4-6 weeks old). Teratomas were formed at around 2 month after the injection. After the formation of the teratoma, mice were euthanized and tumors were dissected for further histological analysis. The related animal protocol was approved by the Institutional Animal Care and Use Committee.
Motor neuron and forebrain neuron differentiation from hPSC lines

Stem cells were cultured on a feeder layer of irradiated MEFs with the hESC media (+10 ng/ml FGF-2) changed daily. To generate spinal motor neurons from hPSCs (Chen et al., 2014; Du et al., 2015), hPSCs were first differentiated to neuroepithelia in a neural medium consisting of DMEM/F12, N2 supplement, and nonessential amino acids in the presence of SB431542 (2 μM), LDN193189 (300 nM), and CHIR99021 (3 μM) for 7 days. At day 8, the neuroepithelia were treated with RA (0.1 μM) and Purmorphamine (0.5 μM) for spinal motor neurons induction (Chen et al., 2014). For generation of forebrain neurons, RA and Purmorphamine were not added. At day 14, spinal motor neuron progenitors in the form of rosettes were isolated and expanded as floating clusters in suspension in the same respective medium but without SB431542, LDN193189 and CHIR99021 for additional 7 days before being plated on laminin substrate for the generation of mature neurons. To generate synchronized postmitotic neurons, the cultures were treated from day 18 to 21 with compound E (0.1 μM) to inhibit cell proliferation.

Immunocytochemistry and quantification

Coverslips were fixed with 4% paraformaldehyde and immunohistochemistry was performed as previously described (Li et al., 2005). Antigen-antibody reactions were developed by appropriate fluorescence-conjugated secondary antibodies. Nuclei were visualized by Hoechst staining. Primary antibodies used in this study included mouse anti-Tra-1-60 (1:50 Santa Cruz Biotechnology), goat anti-Nanog (1:500, R&D), mouse anti-SSEA-4 (1:100, Developmental Studies Hybridoma Bank, DSHB), mouse anti-HB9 (1:50 DSHB), rabbit anti-Foxg1 (1:100, Abcam), rabbit anti-Tbr1 (1:1,000, Proteintech), mouse anti-βIII-tubulin (Tuj1, 1:100, DSHB), rabbit anti-Tau (1:200, Sigma-Aldrich). The population of HB9-expressing neurons among total differentiated cells (Hoechst labeled) was counted as described previously (Li et al., 2009). Briefly, the Zeiss microscope was used to capture images. At
least 4 fields of each coverslip were chosen and counted using Image J software. For each group, 6 coverslips were counted. To quantify axonal swellings, blindly-selected fields were imaged from 6 coverslips per group. The number of axonal swellings was counted (at least 500 neurites were analyzed per group) and divided by the total length of Tau$^+$ axons in each field, which were measured using Image J software as we described before (Denton et al., 2014).

**DNA, RNA isolation, PCR, and RT-qPCR**

Total RNA was extracted from cultures at different stages using TRIzol, treated with DNase to remove genomic DNA according to the supplier’s protocol, and used as templates for the RT-qPCR reaction. To examine the mRNA expression of SMN-FL, quantitative PCR (qPCR) reactions were performed in a 20-μl mixture containing cDNA, primers, and 1× SYBR GREEN PCR Master mix (Bio-Rad). Standard curves and melting curves were plotted for each set of primers to confirm that only 1 amplicon was generated at the same efficiency as GAPDH, a housekeeping gene. Expression levels of the mRNA were calculated using the comparative $C_T$ method. The following primers were used: SMN-FL, 5’-ATGTTAATTTCATGGTACATG-3’, 5’-GGAATGTGAGCACCTTCCTTC-3’; GAPDH, 5’-ATGACATCAAGAAGGTGGTG-3’, 5’-CATACCAGGAAATGAGCTTG-3’. To examine the expression of $SMN1$ gene, DNA was isolated from iPSC stage using ZR Genomic DNA II Kit according to the supplier’s protocol, and PCR was performed. The specific primer sequences used for $SMN1$ were SMN1ex7F: 5’-TTCCTTTATTTTACGGGTGTC-3’, SMNex8R: 5’-CTACAACACCCTTCTCACAG-3’.

**Western blot**

Cell pellets were collected and resuspended in lysis buffer with protease inhibitor cocktail (Sigma), and then passed through a 28.5-gauge needle and lysed. The particulate fraction was
removed by centrifugation. Proteins (10-20 μg) were separated on 10% SDS-PAGE and subjected to immunoblotting analysis. Both blocking and antibody incubations were carried out in Tris-Buffered Saline Tween-20 buffer (TBST, 10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20, pH 8.0) containing 5% nonfat dry milk. Primary antibodies used were rabbit anti-actin (1:1000, Sigma) and mouse anti-SMN1 (1:1000, Abnova). Horseradish peroxidase-conjugated secondary antibodies were detected with Western Lighting Chemiluminescence Reagent Plus (Pierce). For quantifying the SMN1 protein, SMN band intensities were normalized with actin and compared between different groups using ImageJ.

**Live Cell Imaging with MitoTracker**

Spinal motor neuron progenitors were plated onto polyornithine and laminin coated 35mm dishes (MatTek). At day 24 of total differentiation, the cells were stained with 50 nM MitoTracker Red CMXRos (Invitrogen) for 3 minutes to allow visualization of mitochondria and then replaced with fresh medium. Live-cell imaging was performed using a Carl Zeiss Axiovert 200M microscope equipped with an incubation chamber. Axons identified according to morphological criteria (constant thin diameter, long neurites, no branching and direct emergence from the cell body) were imaged every 5 seconds for 5 minutes, yielding 60 frames. Because of photobleaching, the exposure time and light intensity were carefully adjusted (~500ms, 25% light intensity) so that signal was not bleached by the end of 5 minutes of imaging time. Quantifications were performed using the same protocol as described previously (De Vos and Sheetz, 2007; Denton et al., 2014). In short, the location of each mitochondria was manually selected using the Track Points function in MetaMorph, and parameters such as distance from cell body and velocity were recorded. A velocity threshold of 300 nm/s was used to select microtubule based transport events (De Vos and Sheetz, 2007).
Mitochondria that changed position (velocity >300 nm/s) in at least 3 consecutive frames were considered motile.

To analyze mitochondrial morphology, the same straightened images that were generated for measuring mitochondrial transport were used. Within ImageJ, we set the scaling of the image to match the objective used, and then the threshold function was used so that all of the mitochondria were highlighted. Next, the analyzed particles function was utilized with the following conditions (size=0.2-Infinity; circularity= 0-1; show= Ellipses). We measured the length of each imaged axon, and divided it by the number of mitochondria within the region to analyze the mitochondrial density (mitochondria/μm axon). To analyze the mitochondrial area (mitochondria/μm²), the total mitochondrial area was measured and divided by the number of mitochondria within the region.

**Measurement of mitochondrial membrane potential**

Mitochondrial membrane potential was measured based on a previous protocol (Joshi and Bakowska, 2011). Neurons were plated on 35mm glass-bottomed dishes. The Fluorescent dye tetramethylrhodamine methyl ester (TMRM, Invitrogen) was used because it accumulates in mitochondria based on Δψm. Cells were washed three times with 5mM k⁺, 2mM Ca²⁺ Tyrodes solution, then incubated with 10nM TMRM in 2 ml Tyrodes solution for 45 min at room temperature in the dark. Living image was performed using a Zeiss Axiovert 200M microscope equipped with an incubation chamber, using an EC plan-Neofluar 40x1.30 Oil DIC objective. The cells were kept at 37 °C with 5%CO₂ while imaging. Microscope settings were optimized using control cells, and these settings were used for all other groups. Randomly selected fields were imaged every 20s for a total 600s. The mitochondrial uncoupler FCCP was added to the media after 300s and the final concentration was 1 μM. The TMRM fluorescence intensity before and after FCCP was analyzed using Metamorph software and at least 20 regions of interest (ROIs) were traced around mitochondrial
structures for each cell, along with adjacent background regions. The pixel intensity for each region was determined followed by background subtraction.

**Analysis of caspase 3/7 activity**

For measurements of the activities of caspase3 and 7, the Caspase-Glo 3/7 Assay (Promega) was carried out according to the manufacturer’s instructions. Briefly, spinal motor neuron cultures were dissociated with Accutase (Invitrogen) and seeded into 96-well plates at 5000 cells/well in 50 μl of caspase-3/7 reagent. After incubation for 1h at room temperature, luminescence from each well was then measured using Wallac Victor2 1420 MultiLabel Counter.

**Statistical analysis**

The statistical significance of mean differences among different sample groups was analyzed using two-sided Student’s t-test. The significance level was defined as $P < 0.05$. 
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COMPETING INTERESTS

The authors declare that they have no competing financial interests.

AUTHOR CONTRIBUTIONS

C.X., K.D., and X.L. designed the study. C.X., K.D., and Z.W. performed the experiments. C.X., K.D., X.Z., and X.L. analyzed the data. C.X. and X.L. wrote the paper with comments from K.D., Z.W., and X.Z.
References


Fig. 1. Generation and characterization of WT and SMA iPSCs. (A) WT and SMA Type1 iPSC lines expressed the pluripotency markers NANOG, SSEA4 and TRA-1-60. Blue indicated Hoechst stained nuclei. Scale bars: 100 μm. (B) Hematoxylin and eosin staining of teratoma sections that were derived from iPSCs. Tissues from each germ layer were formed. Scale bars: 50 μm. (C) The SMA iPSC lines maintained a normal 46, XY male karyotype after 10 passages as shown by G-banded analysis. (D) qPCR analysis revealed a significant decreased expression of SMN-FL mRNA in SMA Type1 iPSC lines, n=3. (E) PCR analysis showed the absence of SMN1 gene in genomic DNA samples in SMA Type1 iPSC lines. (F) Western blot analysis revealed a significant decrease in SMN1 protein in SMA iPSCs compared to WT. Data presented as mean ± SD. *P < 0.05 versus WT.
Fig. 2. Differentiation of spinal motor neuron from SMA type1 and WT iPSCs. (A) Representative phase contrast images of WT control and SMA type1 iPSCs during neural differentiation at various stages. EB, embryoid body; NEP, neuroepithelial progenitor; MNP, motor neuron progenitor; MN, motor neurons. Scale bar: 100 μm. (B) Immunostaining showing the generation of HB9+ spinal motor neurons from WT and SMA type1 iPSCs at day 24 after differentiation. Blue indicated Hoechst stained nuclei. Scale bars: 20 μm. (C) Quantification of HB9+ neurons did not reveal any significant differences between SMA and WT groups. Data are presented as mean ± SD, n=6.
Fig. 3. Decreased mitochondrial axonal transport in SMA spinal motor neuron cultures.

(A) Representative distance versus time kymographs showing mitochondrial transport in day 24 spinal motor neurons. Scale bar: 10 μm. (B) The percentage of motile mitochondria was decreased in SMA Type1 spinal motor neurons cultures. The percentage of motile mitochondria was significantly increased after treatment with NAC. (C) The number of motile events per mitochondrion was also significantly decreased in SMA Type1 spinal motor neurons. (D and E) Velocities of movement events in retrograde (D) and anterograde (E) directions were not affected in SMA spinal neurons. (F) The frequency of motile movement events (per mitochondrion over 5 min) in the retrograde direction was significantly decreased, whereas the frequency of events in the anterograde direction (G) was non-significantly reduced in SMA spinal motor neurons. Data are presented as mean ± SEM, n=10-20 cells. *P < 0.05 versus WT, #P < 0.05 versus SMA Type 1.
Fig. 4. Mitochondrial morphology abnormalities in spinal motor neuron cultures. (A) Representative images of mitochondria in day 24 spinal motor neurons axon from WT, SMA type 1 and SMA type 1 NAC treated groups. Scale bars: 5μm. (B) The number of mitochondria was decreased in SMA Type 1 derived spinal motor neurons compared with WT. NAC, a potent antioxidant, was applied to neural cultures derived from SMA Type 1 iPSCs at day 13 after differentiation. The number of mitochondria was significantly increased after treatment with NAC. (C) There were no significant differences in the length of mitochondria between different groups. (D) The mitochondria area was significantly decreased in SMA neurons compared with WT. Data presented as mean ± SEM. n=10-20 cells. *P < 0.05 versus WT, #P < 0.05 versus SMA Type 1.
Fig. 5. Mitochondrial morphology and transport in SMA forebrain neuron cultures. (A) Immunostaining showing the generation of telencephalic progenitors (FOXG1+) and subsequent TBR1+ glutamatergic forebrain neurons (TBR1+ cells doubled with TUJ1, a
neuronal marker) from iPSCs. Scale bars: 50μm. (B) Representative images of mitochondria in day 24 forebrain neuron axons from WT and SMA type 1 groups. Scale bars: 5μm. (C) Quantification of the percentage of motile mitochondria. (D) Quantification of the motile events per mitochondrion (velocity > 300nm/s). (E and F) The velocities of movement events in retrograde (E) and anterograde (F) directions were not affected in SMA forebrain neuron cultures. (G and H) The frequency of motile events in the retrograde (G) and anterograde directions (H) was not altered in forebrain neuron cultures. (I) Quantification of the number of mitochondria in SMA forebrain neuron cultures. There were no significant differences in the length (J) and area (K) of mitochondria in SMA forebrain neuron cultures between different groups. Data are presented as mean ± SEM, n=10-20 cells.
Fig. 6. NAC rescued the motor neuron-specific degeneration in SMA long-term cultures.

(A) Tau immunostaining showing axons of 6-week-old forebrain (FB) neuron cultures, spinal motor neuron (MN) cultures, and spinal motor neuron cultures treated with NAC (MN + NAC). Boxed areas are enlarged in insets. (B) Quantification revealed a significant increase in axonal swellings in SMA patient-derived neurons compared to the WT. The number of axonal swelling was significantly decreased after treatment with NAC. (C) The activity of caspase 3/7 was significantly increased in motor neuron cultures derived from SMA Type 1 iPSCs, and this increase was significantly inhibited by the application of NAC. Data are presented as mean ± SEM, n=6-7. * P < 0.05 versus WT group, # P < 0.05 versus SMA Type 1 group. Scale bar: 50 μm.
Fig. 7. Protective effects of NAC in spinal motor neuron cultures derived from SMA Patient 2 iPSCs. (A) SMA iPSCs derived from a different type 1 patient (Patient 2), as well as control iPSCs, were differentiated into spinal motor neurons. At 6 weeks after differentiation (day 42, D42), Tau immunostaining revealed the presence of swellings along axons in SMA spinal motor neuron cultures. (B) Quantification revealed a significant increase in axonal swellings in patient 2-derived neurons, which was mitigated by the treatment of NAC. Data are presented as mean ± SEM, n=6 coverslips, with at least 500 axons being analyzed per group. (C) The activity of caspase 3/7 was significantly increased in SMA Patient 2 iPSC-derived motor neuron cultures (D42); the increased activity was significantly inhibited by the application of NAC. Mean ± SEM, n=5. (D) To examine the mitochondrial membrane potential, the fluorescence intensity of potential dependent dye
TMRM was measured in iPSC-derived spinal motor neuron cultures (day 36) in WT, SMA Patient 2, and SMA Patient 2 plus NAC treated groups. (E) Quantification revealed a significant increase in TMRM fluorescence intensity in SMA Patient 2-derived spinal motor neurons, which was rescued by the treatment of NAC. Data are presented as mean ± SEM, n=3 coverslips, with at least 50 cells being analyzed per group. ** $P < 0.01$ versus WT group, # $P < 0.05$ versus SMA Type 1 group. Scale bar: 50 μm.
**TRANSLATIONAL IMPACT**

**Clinical issue**

Spinal muscular atrophy (SMA), the leading genetic cause of death among infants and toddlers, is caused by a decreased level of functional survival motor neuron (SMN) protein, which leads to the specific degeneration of spinal motor neurons and subsequent muscle atrophy. Currently, there is no effective treatment to prevent or reverse motor neuron degeneration in SMA. Even the fundamental question why spinal motor neurons are specifically affected by the deficiency of SMN, a ubiquitously expressed protein, is still not clear.

**Results**

Here, using SMN-knockdown hESCs and SMA patient-specific iPSCs, the authors examined the role of SMN on mitochondrial transport and morphology in human SMA spinal motor neurons. The initial differentiation efficiency of spinal motor neurons from SMA iPSCs was not affected. After long-term culture, SMA spinal motor neurons, but not forebrain neurons, exhibited increased axonal swellings and elevated caspase 3/7 activity, recapitulating motor neuron-specific degeneration in SMA patients. Using this SMA human model, the authors observed a significant reduction of mitochondrial transport, number, and size in axons of SMA spinal motor neurons at an early stage before motor neurons degenerated. Notably, the abnormal mitochondrial defects were only observed in spinal motor neurons, but not forebrain neurons which are not affected in the disease, revealing the cell-type specific defects. Moreover, motor neurons derived from SMN-knockdown hESCs exhibited similar abnormal mitochondrial dynamics as those from SMA iPSCs, confirming the direct link between these mitochondrial defects and SMN deficiency. Finally, the application of N-
acetylcysteine ameliorated mitochondrial morphology and transport defects, improved mitochondrial health, and rescued subsequent motor neuron degeneration in SMA cultures.

**Implications and future directions**

This study identifies abnormal mitochondrial transport and morphology as early pathological changes in human SMA models, which are implicated in the motor neuron-specific degeneration in SMA patients. Importantly, these early mitochondrial defects serve as a potential therapeutic target for rescuing motor neuron degeneration in SMA. These findings are broadly relevant to the field of neurodegenerative diseases since mitochondrial defects have been reported in many other common neurological diseases including Parkinson’s disease, Amyotrophic Lateral Sclerosis, and Alzheimer’s disease. A deeper understanding of how and why mitochondrial dynamics and function are impaired in patients-derived neurons will provide valuable insights into identifying potential therapeutics for these neurodegenerative disorders.