Stathmin, a microtubule-destabilizing protein, is dysregulated in spinal muscular atrophy

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Spinal muscular atrophy (SMA), a motor neuron degeneration disorder, is caused by either mutations or deletions of survival motor neuron 1 (SMN1) gene which result in insufficient SMN protein. Here, we describe a potential link between stathmin and microtubule defects in SMA. Stathmin was identified by screening Smn-knockdown NSC34 cells through proteomics analysis. We found that stathmin was aberrantly upregulated in vitro and in vivo, leading to a decreased level of polymerized tubulin, which was correlated with disease severity. Reduced microtubule densities and βIII-tubulin levels in distal axons of affected SMA-like mice and an impaired microtubule network in Smn-deficient cells were observed, suggesting an involvement of stathmin in those microtubule defects. Furthermore, knockdown of stathmin restored the microtubule network defects of Smn-deficient cells, promoted axon outgrowth and reduced the defect in mitochondria transport in SMA-like motor neurons. We conclude that aberrant stathmin levels may play a detrimental role in SMA; this finding suggests a novel approach to treating SMA by enhancing microtubule stability.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive disorder characterized by degeneration of spinal cord motor neurons, leading to muscle atrophy and paralysis (1). Childhood SMA is commonly divided into three types according to the severity of symptoms and age of onset (2). All three types of SMA are caused by deletions or mutations of SMN1, the telomeric copy of the SMN gene (3). The centromeric copy of the SMN gene, SMN2, is nearly identical to SMN1 but can be distinguished by nucleotide differences within exons 7 and 8 (3–5). However, SMN2 cannot functionally substitute for SMN1, as SMN1 produces >90% of full-length mRNA, whereas most SMN2 transcripts are missing exon 7 mRNA and encode a C-terminal-truncated SMN protein (4). SMN levels correlate with disease severity (6,7), as confirmed in SMA mouse models (8–11).

The SMN protein is ubiquitously expressed in all tissues and is found in both the cytoplasm and the nucleus (12,13). SMN is involved in the assembly and maturation of spliceosomal small nuclear ribonucleoprotein (12,14–17). However, the mechanisms by which SMN deficiency causes motor neuron degeneration remain to be elucidated. Over the past few years, several findings, such as the fact that SMN localizes in neurites, growth cones and branch points of motor neurons (18–21), indicate its specific involvement in neuronal processes. Motor neurons that lack SMN have shorter axons, smaller growth cones and defects in axonal β-actin mRNA transport, which suggest that SMN plays an important role in the maintenance of motor neurons (22,23).

Stathmin/Op18 is a ubiquitous phosphoprotein with high expression in the developing nervous system (24) and which is downregulated following terminal differentiation (25,26). Stathmin promotes microtubule depolymerization by increasing the microtubule catastrophe rate in vivo (27). Phosphorylation of stathmin at serine residues 16, 25, 38 and 63 is induced by a number of extracellular signals, leading to a loss of the microtubule-destabilizing activity (28–33).
In the present study, we identify stathmin as a detrimental factor in SMA through proteomic analysis. The upregulation of stathmin is correlated with the severity of microtubule defects identified in SMA conditions. Further knockdown of stathmin expression reduced the microtubule defects and contributed to axon outgrowth in SMA-like motor neurons. According to these findings, stathmin is possibly a pathogenic factor in SMA, and the disruption of microtubule maintenance may be partly involved in SMA pathogenesis.

RESULTS

Stathmin is differentially expressed in Smn-knockdown NSC34 cells and in SMA-like mice

To identify potential factors which may be differentially regulated in SMA motor neuron, we generated inducible Smn-knockdown NSC34 cells that mimic the SMN-deficiency condition. Smn protein was successfully knocked down by treating cells with doxycycline (Dox) in a time-dependent manner (Fig. 1A shows three different stable clones, C.21, 22 and 24); in contrast, no changes in Smn levels were observed in vector-transfected control cells (Fig. 1A, Cont.). After 96 h Dox treatment, the Smn level in our inducible knockdown system could be decreased to ~60–75% (Fig. 1B), and total cell lysates were then extracted from Smn-deficient and control NSC34 cells and analyzed by 2D SDS–PAGE (Supplementary Material, Table S1). Seven differentially expressed protein spots were identified by MALDI-Q-TOF mass spectrometry (Supplementary Material, Table S1). One of the spots upregulated in Smn-knockdown NSC34 cells was the well-known microtubule-destabilizing factor, stathmin. To confirm the upregulation of stathmin, stathmin levels in total protein extracts from the stable Smn-knockdown clones were analyzed by western blot. Stathmin levels were indeed higher in Dox-treated Smn-knockdown cells than in non-treated cells. Figure 1C shows the results with clone 21, and consistent results were also found with the other Smn-knockdown clones (data not shown). Stathmin levels increased in a time-dependent manner (data not shown). Stathmin levels increased in severe SMA-like mice but not in type III SMA-like mice. *P = 0.0252, **P < 0.0001 when SMA-like mice were compared with control littermates by t-test. n.s., not significant. (F) Spinal cord samples from nine pairs (n = 3 in each SMA type) of SMA-like mice and control littermates were collected at P12–P14. Type II SMA-like mice and their control littermates were collected at P12–P14. Type III SMA-like mice and their control littermates were collected at about 6–8 weeks after birth. (G) Stable cell lines carrying Smn-specific shRNA were treated with (Smn KD, right) or without (control, left) Dox for 5 days followed by transfection with 3 μg FLAG-stathmin for 16 h. After CHX treatment, cell lysates (n = 6 in each group) were collected at indicated time points and FLAG-stathmin levels analyzed by western blot. Error bar represent SD.
manner in Dox-treated cells and were correlated with a continuing decrease in Smn levels (Fig. 1C, right panel). There was no change in stathmin levels in the control stable clone with or without Dox treatment (Fig. 1C, left panel). We then examined whether the upregulation of stathmin levels could be found in vivo. Total protein extracts from SMA-like mice and control mice were analyzed by western blot. Stathmin levels were upregulated specifically in the spinal cord (1.7 times higher than control, \( P < 0.0001 \)) and in sciatic nerves (1.3 times higher than control, \( P = 0.0252 \)) of SMA-like mice in comparison with control littersmates, but there was no significant difference in stathmin levels in the brain (Fig. 1D and E).

To further address whether the upregulated stathmin levels may correlate with disease severity, we analyzed stathmin levels by western blot. Low stathmin levels were associated with normal developmental progression in control littersmates (Fig. 1F). However, stathmin levels were upregulated in type I (left panel, 1.12 ± 0.14 versus 0.59 ± 0.05; \( P = 0.0273 \)) and type II SMA-like mice (middle panel, 0.76 ± 0.06 versus 0.47 ± 0.06; \( P = 0.0294 \)) in comparison with control littersmates. There was no significant difference in stathmin levels in type III SMA-like mice (right panel, 0.43 ± 0.05 versus 0.43 ± 0.04).

Next, we asked whether stathmin upregulation may be caused by transcriptional activation of *stathmin* gene or by enhancement of its stability under SMA conditions. Spinal cord samples were isolated from type I SMA-like mice, and *stathmin* RNA levels were then evaluated through real-time RT–PCR. However, there was no significant difference in *stathmin* expression (Supplementary Material, Fig. S6). We then tested stathmin stability in *Smn*-knockdown NSC34 cells. Before cycloheximide (CHX) treatment, FLAG-*stathmin* expression vector was transfected into NSC34 cells for 16 h. Cell lysates were then harvested at indicated time points. In *Smn*-knockdown groups, the amount by which stathmin levels dropped, compared with control, was moderate (Fig. 1G, 81.1 ± 11 versus 78.8 ± 8.9% at 2 h; 69.1 ± 9.8 versus 57 ± 10.2% at 4 h; 56.3 ± 10.2 versus 38.5 ± 7% at 6 h; 53.3 ± 10.4 versus 32.8 ± 9% at 8 h). The half-life of FLAG-stathmin protein was increased in *Smn*-knockdown cells (\( t_{1/2} = 7.71 \)) in comparison with control cells (\( t_{1/2} = 5.31 \)), demonstrating that stathmin stability was enhanced (Fig. 1G). Taken together, these results show that stathmin is upregulated in SMA-like mice and this may be correlated with the severity of SMA.

**Impaired microtubule network in Smn-deficient cells**

Since stathmin upregulation was identified in the SMA condition, we then sought to determine whether abnormal stathmin expression could affect microtubule polymerization. After Smn was knocked down for 5 days, we measured the amounts of total α-tubulin and of specific tubulin polymer by western blot. There was no difference in the total α-tubulin levels when normalized to GAPDH levels (0.33 ± 0.06 versus 0.33 ± 0.05 in control; 0.32 ± 0.01 versus 0.33 ± 0.03 in clone 21; 0.33 ± 0.05 versus 0.32 ± 0.07 in clone 22; Fig. 2A and B), but the proportion of tubulin polymers as a fraction of total tubulin fell by ~13–17% in *Smn*-knockdown cell lines compared with cells without Dox treatment (49.7 ± 1.6 versus 43.3 ± 1.67% in clone 21; 48.4 ± 1.7 versus 39.9 ± 1.6% in clone 22; Fig. 2D). In contrast, the control line stably transfected with empty vector showed no change in polymerized tubulin levels with or without Dox treatment (50 ± 3.9 versus 48.8 ± 3.5%; Fig. 2D), demonstrating that Dox itself does not affect the microtubule network. To confirm the specificity of the detection system, we treated control cells with nocodazole or paclitaxel before harvesting. In paclitaxel-treated cells, most of the α-tubulin signals in pellet fractions were due to polymerized tubulin; in contrast, nocodazole-treated cells showed tubulin signals predominantly in the soluble fraction (Fig. 2C).

Possible defects in the microtubule network in *Smn*-knockdown cells were further examined by microtubule re-growth experiments. Cells were pretreated with nocodazole for 6 h to depolymerize microtubules, and images immunostained for α-tubulin were then acquired at 0, 30 and 60 min after nocodazole washout. There was no detectable difference in the control stable clone with or without Dox treatment (Fig. 3A). In *Smn*-knockdown cells, microtubule polymerization was initially normal, but by 60 min, the formation of an extended microtubule network was impaired compared with cells without Dox treatment (Fig. 3B). Together, our findings indicate that microtubule polymerization is disrupted in Smn-deficient NSC34 cells.

**Knockdown of stathmin rescues the impaired microtubule network in *Smn*-knockdown cells**

Tubulin polymerization was affected in *Smn*-knockdown cells (Figs 2D and 3B). We therefore asked whether this may be caused by an upregulation of stathmin. To test this possibility, we introduced stathmin siRNA into Smn-deficient NSC34 cells to see whether this could restore the impaired microtubule network. Stathmin was efficiently knocked down by transient transfection with *stathmin* siRNA in NSC34 cells (Fig. 4A). At 72 h after transfection, cells were incubated with nocodazole for a further 6 h, and images were then acquired at 0, 30 and 60 min after nocodazole washout. To visualize siRNA in cells, siRNAs were labeled with a fluorescent Cy3-conjugated dye. In *stathmin* siRNA-transfected cells, downregulation of endogenous stathmin expression did not recover nocodazole-induced microtubule network destruction (Fig. 4Bd); however, it did promote the integrity of microtubule network formation in comparison with control siRNA-transfected cells (Fig. 4Bc and f). This finding demonstrates that the reduction of stathmin expression in *Smn*-knockdown cells ameliorated the microtubule network defect caused by Smn deficiency.

**Abnormality of microtubule in SMA-like mice**

Stathmin upregulation was originally detected in motor neuron-like NSC34 cells. Since aberrant stathmin expression was found here (Fig. 1) in spinal cords and sciatic nerves of type I SMA-like mice, we further examined whether this exhibited any cell-type specificity. Sections of lumbar spinal cords from type I SMA-like mice and their control littersmates were immunostained using antibodies for stathmin and the motor neuron marker, ChAT. Surprisingly, there was significant stathmin accumulation in spinal cord ChAT-positive motor neurons of...
SMA-like mice (Fig. 5Ae, indicated by arrows), but not in control mice (Fig. 5Aa, indicated by arrowheads). As stathmin levels were higher in sciatic nerves of SMA mice than those of control littermates (Fig. 1D and E) and stathmin was strongly associated with the microtubule defect in vitro (Figs 3 and 4), we examined whether aberrant stathmin levels in motor neurons could influence microtubule formation in SMA-like mice. Sciatic axons were isolated from affected SMA-like mice and control littermates, and the levels of acetylated α-tubulin (polymerized tubulin marker in axon) and neuronal βIII-tubulin of the sciatic nerves were analyzed by western blot. The amount of assembled tubulin was 27% lower in sciatic nerves of SMA-like mice than of control mice (1.70.19 versus 1.240.65; P, 0.0001; Fig. 5B and C). Interestingly, no changes in levels of βIII-tubulin were detected in proximal nerves or in the spinal cord; however, βIII-tubulin levels were significantly lower in distal sciatic nerves of SMA-like mice (Fig. 5D).

On the basis of these findings, we then asked whether there was any effect on the microtubule of distal axons. Axonal microtubule numbers in cross-sections of diaphragm preparations isolated from type I SMA-like mice and their littermate controls were analyzed by electron microscopy. Phrenic motor neuron axons from the control littermates had a mean density of 14.5 ± 0.3 microtubules/μm² cross-sectional axonal area. In contrast, the affected type I SMA-like mice showed a loss of ~26% in the average number of microtubules per axon (14.5 ± 0.3 versus 10.7 ± 0.3; P < 0.0001; Fig. 6A and B).

To investigate the potential involvement of stathmin in microtubule defects in SMA-like mice, we cultured motor neurons from SMA-like mice and littermate controls to determine neuronal microtubule integrity after 7 days of plating. Immunolabeling for acetylated α-tubulin and tyrosinated α-tubulin (newly depolymerized tubulin) revealed neurite swellings in SMA-like motor neurons (Supplementary Material, Fig. S2). Interestingly, abnormal accumulation of tyrosinated α-tubulin and stathmin was found in these neurite swellings but not in control motor axons, suggesting that the abnormal expression of stathmin may result in microtubule disorganization in SMA-like mice. Taken together, these findings provide a link between stathmin and the microtubule abnormality in SMA-like mice.

**Downregulation of stathmin rescues axonal defects in SMA-like motor neurons**

Recent studies have demonstrated that motor neurons have reduced axonal length in SMA mouse models (19,23); we therefore examined whether knockdown of **stathmin**

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**Figure 2.** Polymerized tubulin level is decreased in Smn-deficient NSC34 cells. (A) Stable cell lines carrying Smn-specific shRNA were induced with 1 μg/ml Dox for 120 h, and Smn levels analyzed by western blot. GAPDH was used as a loading control. (B) The y-axis indicates the α-tubulin level normalized to GAPDH level. Error bars represent SD. ‘Cont.’ indicates control group. C21 and C22 indicate clones 21 and 22, respectively. (C) NSC34 cells were treated with 10 μM nocodazole or 10 μM paclitaxel for 4 h. Cell lysates were analyzed for the amount of α-tubulin in polymerized (P) and soluble (S) fractions by western blot. (D) The amounts of α-tubulin in polymerized form are represented as percentages of the total tubulin levels. The tubulin levels were normalized to GAPDH levels. No significant difference in polymerized α-tubulin level was detected in control cells with or without Dox treatment (labeled as C), but there was a significant decrease in Smn-knockdown clones (labeled as C.21 and C.22). Values represent means ± SD from three independent experiments. ***P < 0.0001 when compared with cells with or without Dox treatment by t-test. n.s., not significant.
expression in SMA-like motor neurons could promote axon outgrowth. Primary dissociated cultures of anterior horn motor neurons were isolated from SMA-like mice (SMN2 copy number 3) (23), and either *stathmin* or control siRNAs were transfected into the motor neurons at 3 days in culture. After motor neurons were immunostained for neurofilament H (NF-H) on day 5, the mean axon length in *stathmin* siRNA-transfected motor neurons was 142.7 ± 5.9 μm, significantly
longer than the control group (95.3 $\pm$ 5.9 $\mu$m; Fig. 7A–C). These data suggest that stathmin knockdown acts beneficially to diminish the axon outgrowth defect in SMA-like motor neurons. In addition, we examined whether the microtubule disruption from abnormal stathmin could influence organelle transport in axons. Primary cultured motor neurons (5 days in culture) were stained by MitoTracker Red for 30 min, fixed and images acquired to determine the mitochondrion density along axons. There was a significant decrease of mitochondrion density in SMA-like motor neurons (16 $\pm$ 1 versus 23 $\pm$ 1.1 100 $\mu$m$^{-1}$ in control motor neurons), suggesting that the upregulation of stathmin also disrupts the transport of mitochondria into axon (Fig. 8A and B). To further confirm that this disruption was directly correlated with stathmin upregulation, stathmin-siRNA was used to knock down stathmin expression in SMA-like motor neurons, and the mitochondria density was then determined. In this case, there was a significant recovery of mitochondrion density in stathmin-siRNA-transfected SMA-like motor neurons (Fig. 8C and D, 19 $\pm$ 1 in test versus 14 $\pm$ 1 100 $\mu$m$^{-1}$ in control siRNA-transfected SMA-like motor neurons), which strengthens the idea that stathmin plays a detrimental role in organelle transport under SMA.

**DISCUSSION**

In this study, we identified that stathmin, a well-studied microtubule-stabilizing protein, is dysregulated in SMA. Knockdown of stathmin expression enhanced microtubule re-growth in Smn-deficient cells and diminished the axonal transport defects in SMA-like motor neurons. These findings support the idea that stathmin may be a detrimental factor in SMA.

It is not yet clear whether defects in microtubule polymerization may contribute to the disease progression of SMA. However, much evidence has accumulated that suggests a direct link between microtubule stabilization and neurodegeneration. Model progressive motor neuropathy (pmn) mice with homologous mutation in the tubulin-specific chaperone E (Tbce) gene develop progressive motor neuron degeneration disease (34). The destabilized Tbce protein causes decreased tubulin levels and obvious loss of microtubule density in distal axons and leads to death of these mice within 4 to 5 weeks after birth (35). Here, we found a 26% loss of axonal microtubules in phrenic nerves of affected type I SMA-like mice, a 27% reduction of polymerized tubulin in sciatic axons and decreased neuronal $\beta_{III}$-tubulin levels in the distal

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**Figure 4.** Knockdown of stathmin restores microtubule re-growth in Smn-deficient NSC34 cells. (A) NSC34 cells were transfected with stathmin or control siRNA, and stathmin levels analyzed by western blot. β-Actin was used as a loading control. (B) Smn-knockdown cells (clone 21) were transiently transfected with control (upper panel) or stathmin (lower panel) siRNA (red) for 72 h, and microtubule re-growth was analyzed at 0, 30 and 60 min after nocodazole washout. Microtubules were stained for α-tubulin (green), and DAPI was used to stain nuclei (blue). Higher magnifications of the areas in the white squares in c and f are shown in the panels at the right. Scale bars, 10 $\mu$m. Nucleus was stained with DAPI (blue).
sciatic nerve. These observations therefore suggest a link to the muscle weakness and axon degeneration in SMA.

Recently, a wide-spread splicing defect has been found in the SMA mice model that has led to the assumption that SMA is a general splicing defect disorder (36). Since there is no remarkable damage to cell types other than motor neuron, it is possible that some tissue-specific regulators are induced, repressed or dysregulated in the SMA conditions, due to defective splicing regulation or some other unknown mechanisms. We carefully examined the expression level of the stathmin gene in the spinal cord of the most severe SMA-like mice but found no significant differences (Supplementary Material, Fig. S6). However, we found that the stability of stathmin was enhanced in our SMA model (Fig. 1G). In addition, we examined the expression patterns of other tubulin-folding cofactors (Tbc A–D) which are involved in tubulin biogenesis and microtubule dynamics, in spinal cord samples from each type of SMA-like mice. There was no significant difference in the expression of Tbc A and C (Supplementary Material, Fig. S5A and C). Tbc D, on the other hand, was upregulated in type I and II SMA-like mice but downregulated in type III SMA-like mice.
mice (Supplementary Material, Fig. S5D). There was no difference in Tbc B levels in type I and II SMA-like mice but Tbc B levels decreased in type III SMA-like mice (Supplementary Material, Fig. S5B). It is known that Tbc D and E regulate microtubule dynamics through sequestering α- and β-tubulin, leading to the dissociation of microtubules (37). This result therefore suggests that some of the tubulin regulators may be dysregulated in SMA.

Since stathmin protein level is correlated with SMA disease severity (Fig. 1F), we hypothesized that stathmin upregulation may follow the loss of Smn expression and thus influence the maintenance of microtubule integrity. It is known that microtubules consist of either tyrosinated or acetylated α-tubulin. The acetylated α-tubulin comprises the stable structure of the microtubule, whereas tyrosinated α-tubulin is a marker of recently disassembled microtubules in an axon (38,39). In control motor neurons, both tyrosinated α-tubulin and acetylated α-tubulin were present along the axon. In contrast, tyrosinated α-tubulin and stathmin accumulated in axonal beads (Supplementary Material, Fig. S2), implying that elevated stathmin levels led to depolymerization of microtubules, increased levels of free tubulin and the formation of swollen structures along the axon. This result was consistent with a previous study that reported that axonal swellings were found in severe SMA mice and in SMA patients (40). Stathmin depletion blocks nerve growth factor-stimulated neurite outgrowth in PC12 cells (41). In addition, aging stathmin-deficient mice develop axonopathy of the central and peripheral system (42). These findings suggest that stathmin is essential for the integrity of motor neurons. Expressing the constitutively active form of stathmin in Purkinje cells disrupts microtubule organization and causes motor discoordination in transgenic mice (43,44). Drosophila with stathmin mutations shows proper oocyte fate determination but also defects in oocyte terminal differentiation. Lack of stathmin causes a marked reduction of total tubulin levels, suggesting that stathmin is essential for long-term maintenance of the microtubule (45).

In this study, we also provide evidence that mitochondrial transport was also reduced (Fig. 8). This defect was correlated with stathmin upregulation under SMA and demonstrates that microtubule disruption could also impair organelle transport. Collectively, these studies show that proper regulation of stathmin levels is essential for correct microtubule dynamics and is important for neuronal function.

In a previous study of amyotrophic lateral sclerosis (ALS) model mice, Strey et al. (46) found that stathmin accumulation in motor neurons in superoxide dismutase 1 (SOD1<sup>G93A</sup>) mice leads to microtubule disruption and Golgi fragmentation. We also found here that stathmin accumulated in motor neurons of SMA-like mice (Fig. 5A); the dysregulated stathmin that is harmful to motor neurons may thus be a secondary consequence of SOD1 mutation or insufficient SMN levels. Furthermore, there was no significant difference in stathmin levels in spinal cords of SMA-like mice and their control littermates at embryonic day 12.5 (E12.5), but abnormal upregulation was seen from E15 until disease onset (Supplementary Material, Fig. S3). These findings suggest different mechanisms of stathmin regulation in ALS and SMA. Another stathmin family protein, SCG10 (47), which is highly homologous to stathmin, also accumulated in motor neurons in SOD1<sup>G93A</sup> mice (46). We examined SCG10 levels in spinal cords of SMA-like mice from the embryonic stage until disease onset by western blot and found no change in levels of SCG10 at E15.5, but higher

Figure 6. Decreased microtubule densities in SMA-like mice. (A) Transverse ultrathin sections of phrenic nerve were collected from SMA-like mice (b and d) and control littermates (a and c) at P7. Arrows mark microtubules. Scale bars: a and b, 0.5 μm; c and d, 100 nm. (B) Microtubule densities per axon area of type I SMA-like mice and control littermates (n = 6 per group) were calculated. A total of 283 axon sections from control mice and a total of 318 axon sections from SMA-like mice were analyzed. Values represent means ± SEM. ***P < 0.0001 when compared with control mice by t-test.
Figure 7. Knockdown of stathmin reduced the axon outgrowth defect in SMA-like motor neurons. (A) Control siRNA. (B) Stathmin siRNA (a, red) was transiently transfected into SMA-like motor neurons. Motor neuron identity was stained for Hb9 (b, blue). Axonal processes were stained for NF-H (c, green). Nuclei were stained with DAPI (d, white). (C) Axon length was counted 48 h after siRNA transfection into motor neurons. Values represent means ± SEM from four independent experiments (n = 70 cells per group). **P < 0.0001, t-test. Scale bar, 50 μm.
levels from E19.5 until death (Supplementary Material, Fig. S4). These findings suggest that other microtubule
destabilizing factors may also be dysregulated in SMA-like mice, and that increased levels of these proteins may be a
risk to motor neurons. In addition, in the SOD1G93A ALS mouse model, a progressive change in microtubule dynamics
in the spinal cord impairs axonal transport in motor neurons. These mice were administered a microtubule-modulating
agent (noscapine) that reduced microtubule turnover and delayed the disease onset (48). Combined with our finding
that the inhibition of stathmin expression in SMA diminished the SMA phenotype, these results suggest that microtubule
dynamics may be a potential therapeutic target and could provide a novel avenue for SMA therapy. Since our findings
provide novel insights into SMA pathogenesis, the mechanism of stathmin involvement in SMA through disrupting microtu-
bule turnover and whether loss of stathmin function may be of benefit in SMA therapy remain unclear and need to be further
addressed in the future.

MATERIALS AND METHODS

Mice
The SMA-like mice were generated as described previously (8). The type I and type II SMA-like mice (Smn<sup>−/−</sup>, SMN2<sup>−/−</sup>) were generated by crossing type III SMA-like mice lines (Smn<sup>−/−</sup>, SMN2<sup>+/+</sup>) with Smn heterozygous mice (Smn<sup>+/−</sup>). The mice that died within 5–9 days after birth (P) or between P12–17 were categorized as type I and type II SMA-like mice, respectively. Both types I and II SMA-like mice were sacrificed when they became significantly weaker than normal. The type III mice were sacrificed at 6–8 weeks after birth. At this time point, those mice had significantly shorter and necrotic tails.

Antibodies
Antibodies used for immunoblot or immunostaining experiments and their dilutions were as follows: SMN (1:10 000,
BD Transduction Laboratories, Palo Alto, CA, USA), α-tubulin and stathmin (1:1000, Calbiochem, San Diego, CA, USA), β-actin and acetylated α-tubulin (1:1000, Sigma, St Louis, MO, USA), tyrosinated α-tubulin (1:1000, Abcam, Cambridge, UK), ChAT, β-tubulin, GAPDH, NF-H and Hb9 (1:500, Chemicon, Temecula, CA, USA). Fluorochrome-conjugated secondary antibodies were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA).

Constructs

Mouse Smn shRNA plasmid was constructed from two chemically synthesized oligonucleotides, 5'-GATCCCGTATGCT ATAGTACAGAACTTCAAGAGAGTTCTGACTATTAGCT ACTTTTTT-3' and 5'-AGCTTTAAGATGCTTAATA GTAC AGAAGTCTCTAGGTTAGTATAGGGCCA T-3', annealed and inserted at the BglII and HindIII sites of pSuperior.puro vector (OligoEngine, Seattle, WA, USA) to form pSuperior-Smn shRNA. The 0.45 kb fragment of stathmin of mouse coding region was subcloned by PCR using specific primer pairs (forward: 5'-ATGGCATCTTCTGATAATGGCAGCTTCTGATA TTCAAGGT-3'; backward: 5'-GATATCTGAAGCCTCAGT AATCCTCCG-3') into the EcoRI site of the pCMV2-FLAG vector (Sigma).

Cell culture, transfection and stable clone selection

The cell line NSC34 was routinely cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated FBS, 1 mM glutamine and 100 IU/ml penicillin and 100 μg/ml streptomycin (Invitrogen), incubated at 37°C in a 5% CO2 humidified atmosphere. Transfections were performed using LipofectAMINE™2000 (Invitrogen) according to the manufacturer’s recommendations.

To generate inducible RNAi-stable clones, NSC34 cells were first transfected with pDNA6/TR vector (Invitrogen), and individual bleaching (Invitrogen)-resistant clones were selected. Next, the NSC34-TR cell line was transfected with pSuperior-Smn shRNA and single clones selected by adding 1 μg/ml puromycin. Finally, western blot experiments were used to evaluate SMN expression levels in each stable clone.

Isolation and primary culture of motor neurons have been described previously (49,50). Synthetic stathmin siRNA and control siRNA were purchased from Dharmaco (Thermo-Fisher Scientific, Lafayette, CO, USA). To visualize siRNA entering cells, fluorescent labeling of siRNA was performed according to the manufacturer’s instructions (Ambion, Austin, TX, USA). Transfections were performed using siMPORTER (Upstate Biotechnology, Lake Placid, NY, USA) following the manufacturer’s standard protocols.

Stathmin stability analysis

Inducible Smn-knockdown clone (clone 21) was treated with or without 1 μg Dox for 5 days, and then cells (5 x 10^5 cells per well) were plated into six-well culture plates. After overnight culture, cells were transfected with FLAG-stathmin and allowed to grow for 16 h. Cells then treated with CHX (50 μg/ml) and harvested at indicated time points. Equal amounts (20 μg) of total protein extracts were analyzed by western blot to determine FLAG expression. Data were collected from six independent experiments.

Western blot

Mouse tissues or cells were homogenized or lysed with RIPA buffer (150 mM NaCl, 50 mM Tris/HCl at pH 8.0, 2 mM EDTA, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM Na3VO4, 50 mM NaF) supplemented with protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Equal amounts of boiled lystate were run on acrylamide gels and then transferred to PVDF membranes. The membranes were incubated in blocking solution (0.1 M PBS, 5% non-fat milk, 0.2% Tween-20) for 1 h at room temperature and then incubated in the same solution with the appropriate primary or secondary antibodies. Finally, these membranes were visualized with enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, MA, USA). Relative densitometric values in western blots were analyzed with AlphaEaseFC software (Alpha Innotech Corporation, San Leandro, CA, USA).

Immunocytochemistry/immunohistochemistry

NSC34 cells, primary motor neurons grown on glass coverslips, or mouse lumbar spinal cord cryosections (8 μm) were fixed, blocked and incubated with the appropriate primary antibodies, followed by incubation with the appropriate fluorescent dye-conjugated secondary antibodies. Finally, samples were treated with DAPI (Sigma) for 5 min to identify nuclei and then mounted with fluorescence mounting solution (DAKO, Glostrup, Denmark). Confocal images were obtained using an LSM510 Meta laser scanning confocal microscope (Zeiss).

In vivo polymerized tubulin assay and microtubule growth assays

The in vivo polymerized tubulin assays were performed according to the manufacturer’s instructions (Cytoskeleton Inc., Denver, CO, USA). Microtubule growth assays were conducted as described previously (35). Control and Smn-knockdown cells grown on glass cover slips were treated with 20 μM nocodazole (Sigma) for 6 h at 37°C. Cells were then washed with warm culture medium and incubated for a further 0, 30 or 60 min. Cultures were rinsed, extracted, fixed and blocked with 3% BSA in PBS. After incubation with the appropriate primary and secondary antibodies, confocal images were captured on a Zeiss LSM510 microscope.

Electron microscopy

The diaphragms were fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer. After extensive washing in phosphate buffer, the tissues were post-fixed in 1% osmium tetroxide in phosphate buffer. Following dehydration, the tissues were embedded in Spurr resin. Ultra-thin sections were stained with uranyl acetate and lead citrate and
Mitochondrial density analysis

Primary cultures of motor neurons were transfected with either synthetic statmin-siRNA or a control siRNA for 48 h. Mitochondria were then labeled by treating cells with MitoTracker Red (Molecular Probes, Invitrogen) for 30 min. After the staining medium was washed out, cells were fixed following.

REFERENCES


