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ORIGINAL ARTICLE

Hydrogen peroxide decreases the survival rate of HeLa cells with stable knockdown of survival motor neuron protein

過氧化氫降低被穩定減弱SMN蛋白質的HeLa細胞的存活率

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Abstract The mutations of *survival motor neuron* (*SMN*) gene result in spinal muscular atrophy (SMA), a common neurodegenerative disease. Some of the motor neurons undergoing cell death is the predominant characteristic in SMA pathology. However, the viability and sensitivity to stresses of other cell types also need to be determined. In this article, we established HeLa stable cell line with inducible SMN knockdown to study its viability and sensitivity to oxidative stress. SMN knockdown in the HeLa stable cell line was induced by doxycycline. The proliferative and survival rates of SMN knockdown cells with or without hydrogen peroxide (H_2O_2) treatment were determined. Our results showed that the proliferative rate of SMN knockdown cells decreased only slightly compared with that of the cells

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without doxycycline treatment. In contrast, after H_2O_2 reached certain concentrations, the survival rate of SMN knockdown cells decreased significantly. Our data indicate that SMN knockdown alone is not critical to cell viability. However, when SMN knockdown cells are under stress, such as oxidative stress, their survival rate may significantly decrease. Our results will be helpful to prevent the detrimental effect caused by the cell death of non-motor neurons under stress in SMA patients. In addition, the cell model we established can be used to study the mechanism and screen drugs to prevent the detrimental effects in cases of SMA disease.

摘要 *Survival of motor neuron* (*SMN*)基因突變會導致神經退化疾病：脊髓肌肉萎縮症(SMA)。有些運動神經元細胞死亡是脊髓肌肉萎縮症的病理特徵。至於病患運動神經元以外其他類型細胞的存活率及對壓力的敏感度則尚待深入研究，本文中，我們在HeLa細胞中建立誘導性基因減弱SMN以研究這些細胞的存活率及對壓力的敏感度。以doxycycline誘導將HeLa細胞中的SMN基因減弱後，再以H_2O_2處理觀察細胞增殖及存活率。我們的結果顯示SMN基因減弱的HeLa細胞與未加入doxycycline的控制組相比，其細胞增殖率只輕微下降。相反的，細胞若經過H_2O_2處理，SMN基因減弱的HeLa細胞其細胞存活率則顯著下降。我們的結果認為SMN基因減弱並非細胞存活的關鍵因素，但是當細胞遭遇壓力如氧化壓力時，細胞存活率會顯著下降。我們的結果將有助於SMA病人避免壓力造成非運動神經元細胞的傷害。此外，我們所建立的細胞模式將有助於SMA疾病的機制研究及藥物篩選。

Introduction

Spinal muscular atrophy (SMA) is a common autosomal recessive neurodegenerative disease characterized by the degeneration of motor neurons in anterior horns of the spinal cord, which leads to muscular paralysis and atrophy. Molecular studies demonstrated that SMA is caused by reduced expression of survival motor neuron (SMN) proteins, resulting from mutations or deletion in *SMN1* gene in humans [1—3]. SMN proteins are the central component of the SMN complexes, which are necessary for the assembly of Sm proteins onto the small nuclear RNAs to form small nuclear ribonucleoproteins (snRNPs). The snRNPs are the essential components of the pre-mRNA splicing machinery to catalyze the excision of introns in the nucleus [4,5]. Depletion of SMN was reported to affect snRNP assembly capacity and Cajal body formation [6,7]. Myoblast stable cell lines (C2C12) with SMN knockdown showed a decrease in the number of nuclear gems, reduced proliferation with no increase in cell death, defects in myoblast fusion, and malformed myotubes [8].

Because SMA is caused by reduced SMN proteins, gene knockdown is a powerful technique to study the role of SMN in SMA. RNA interference (RNAi)-mediated knockdown of SMN in HeLa cells results in decreased levels of Gemins, such as Gemin 2, and disassembly of Cajal bodies [9,10]. Reduction of Drosophila SMN (dSMN) expression by RNAi significantly increased apoptosis in Drosophila S2 cells [11]. Knockdown of SMN expression by RNAi also induced apoptosis in differentiated P19 neural stem cells [12]. SMN knockdown in PC12 cells changed the expression pattern of Profilin II, resulting in altered cytoskeletal integrity and a subsequent defect in neuritogenesis [13]. The short interfering RNA (siRNA)-treated motor neuron—like NSC-34 cell line also showed decreased cell viability [14]. In addition, motor axon—specific pathfinding defects were found in zebrafish embryos treated with antisense morpholinos to reduce the SMN levels [15]. However, most of these experiments treated the cells with siRNA by transient expression system, which makes it difficult to carry out further studies. In this article, we established an inducible stable SMN knockdown system in HeLa cells to study the viability of cells under reduced SMN protein level.

Published neuropathological data indicated that some of the motor neurons in SMA patients die by necrotic or apoptotic cell death [16]. Reactive oxygen or nitrogen species were reported to cause cell death by non-physiological (necrotic) or regulated (apoptotic) pathways [17]. In this article, we established HeLa stable cell line with inducible SMN knockdown to study its viability and sensitivity to oxidative stress. Our results showed that the proliferative rate of SMN knockdown cells only slightly decreased compared with that of the cells without doxycycline treatment. In contrast, after hydrogen peroxide (H_2O_2) reached certain concentrations, the survival rate of SMN knockdown cells decreased significantly. Our data indicate that SMN knockdown alone is not critical to cell viability. However, when SMN knockdown cells are under stress, such as oxidative stress, their survival rate may decrease significantly. With this cell model, we demonstrate that SMN participates in the cell death under oxidative stress.

Materials and methods

SMN siRNA construct

The SMN siRNA construct is used to knockdown the SMN protein. Double-stranded DNA for siRNAs targeting *SMN* genes were inserted into inducible siRNA expression vector pSuperior.neo (OligoEngine, Seattle, WA). The mRNA sequences targeted by siRNA are modified from those described by Shpargel and Matera [10]. The protocols for pSuperior.neo-si*SMN* construction followed the manufacturer's instructions for pSuperior RNAi system (OligoEngine). Briefly, two complementary oligonucleotides, 5'-GATCCCCGGAGCAAAACTTATC

TGACTTCAAGAGAGTCAGATAAGTTTTGCTCC TTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAGGAGCAAAACTTATCTGACTCTCTTG AAGTCAGATAAGTTTTGCTCCGGG-3' were synthesized, annealed, and inserted into pSuperior.neo between BglII and HindIII sites. The correct clones were verified by double digestions with EcoRI and HindIII. The construct we generated was named pSuperior.neo-si*SMN*. The DNA sequence was verified by sequencing.

Cell culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, and antibiotics. Ten micrograms of both pSuperior.neo-si*SMN* and pcDNA6/TR (Invitrogen, Carlsbad, CA) were cotransfected into HeLa cells by electroporation. The cells were then selected with 500 μg/mL G418 and 2 μg/mL blasticidin for clones with stably transfected pSuperior.neo-si*SMN* and pcDNA6/TR.

Western blotting

Western blotting is used to detect the protein levels of SMN and controlled beta-actin. Cells were harvested and lysed in RIPA buffer (50 mM Tris-HCl, pH 8; 1 mM EDTA; 150 mM NaCl; 1% nonidet P40 (NP40); 0.5% sodium deoxycholate; 1% sodium dodecyl sulfate (SDS), 1× protease inhibitor cocktails; and 1 mM phenylmethylsulfonyl fluoride (PMSF)) at 4°C for 20 minutes. The lysates were subjected to sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. The blots were probed with anti-SMN antibody (BD Biosciences, Frankin Lakes, NJ) or anti-beta-actin antibody (Santa Cruz, Santa Cruz, CA) and detected by ECL chemiluminescence kit (GE Healthcare, Little Chalfont, UK).

Cell proliferation assay

The cell proliferation assay is used to calculate the cell number after proliferation. The selected HeLa cells with doxycycline (Sigma, St. Louis, MO) treatment represented the cells with SMN knockdown. The cells without doxycycline treatment were used as the control. 1×10^4 cells were cultured in 6-cm dishes for 12 hours and then induced with doxycycline (1 μg/mL) each alternate day. The cells were then incubated at 37°C from 0 days to 6 days, and the numbers were counted.

Cell survival assay

The cell survival assay is used to calculate the number of surviving cells after H_2O_2 treatment. The selected HeLa cells with doxycycline treatment represented the cells with SMN knockdown. The cells without doxycycline treatment were used as the control. The cell survival assays were performed by CellTiter 96 Aqueous One Solution (Promega, Madison, WI). The protocols were according to the technical bulletin for CellTiter 96 Aqueous One Solution. Briefly, 1,000 cells were cultured in 96-well plates for 12 hours and then induced with doxycycline (1 μg/mL) for a further 48 hours. The cells were then treated with H_2O_2 for 24 hours. Twenty microliters of CellTiter 96 Aqueous One Solution Reagent was added to each well and incubated at 37°C for 1 hour. Finally, the absorbance of 490 nm was measured.

Results

Inducible knockdown of SMN protein in HeLa cells

To gain insight into the functions of SMN, we tried to establish a cell system with inducible knockdown of SMN protein. Double-stranded DNA for siRNAs targeting *SMN* genes was inserted into inducible siRNA expression vector pSuperior.neo. The construct that we generated was named as pSuperior.neo-si*SMN*. Tetracycline repressor encoded from pcDNA6/TR depressed the expression of siRNAs in the absence of tetracycline or doxycycline. The pSuperior.neo-si*SMN* and pcDNA6/TR were cotransfected into HeLa cells. The cells were then selected with Genetisin (G418) and blasticidin for clones with stably transfected pSuperior.neo-si*SMN* and pcDNA6/TR. Stable cell lines were then treated with doxycycline from 0.1 μg/mL to 1 μg/mL to induce SMN knockdown. It was reported that SMN protein levels decreased after 44—60 hours with SMN RNAi [9,10].

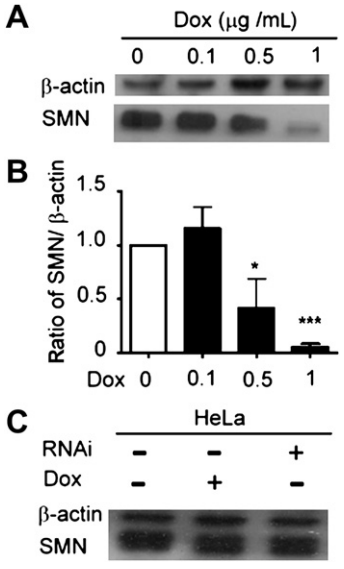

Figure 1. Decreased survival of motor neuron (SMN) protein levels induced by doxycycline in the HeLa stable cell line. HeLa cells were cotransfected with pSuperior.neo-si*SMN* and pcDNA6/TR and then selected by G418 and blasticidin. (A) The selected stable cell line was treated with doxycycline from 0 ug/mL to 1 ug/mL to induce SMN protein knockdown. The protein levels of SMN were analyzed 48 hours after doxycycline treatment by Western blotting. The beta-actin proteins were used as loading control. (B) The protein levels were quantified. The error bars represent standard deviations calculated from three independent experiments. Significant differences between cells with and without doxycycline treatment are shown (*$p < 0.05$, **$p < 0.001$). (C) The protein levels of the HeLa stable cell line without doxycycline treatment and normal HeLa cells with or without doxycycline treatment were analyzed by Western blotting.

The SMN protein levels of stable cell lines were then detected 48 hours after doxycycline treatment by Western blotting with anti-SMN antibody. The SMN protein level in one of the stable clones was knocked down successfully by doxycycline treatment (Fig. 1A). The results showed that the SMN protein levels decreased to 42% and 5.4% of normal cells with 0.5 μg/mL and 1.0 μg/mL doxycycline treatment, respectively (Fig. 1B). This indicated that the extent of SMN knockdown depended on the concentration of doxycycline, and that the best concentration of doxycycline to knock down SMN was 1 μg/mL. We then treated this cell line with 1 μg/mL doxycycline for the remaining experiments. The SMN level of this HeLa stable cell line without doxycycline treatment was similar to normal HeLa cells with or without doxycycline treatment (Fig. 1C). These data demonstrated that we had successfully established a HeLa cell model with inducible knockdown of SMN expression.

The proliferative rate of cells with reduced SMN protein level was not significantly changed

Motor neurons with reduced SMN protein in SMA patients were reported to die by apoptotic or necrotic cell death. We then analyzed the viable cell number to study the viability of the cells we established under SMN knockdown. We treated the HeLa stable cell line with doxycycline each alternate day to decrease the SMN protein level. The proliferative rates of cells were then analyzed by cell number counting. This experiment was repeated three times. The results showed that the proliferative rate of cells with reduced SMN protein level decreased only slightly compared with that of the cells without doxycycline treatment (Fig. 2). The difference between the cells with or without SMN knockdown did not reach a statistically significant level by *T* test analysis. These data indicate that reduced SMN protein level alone may not be critical to cell viability in some cell types, such as HeLa cells.

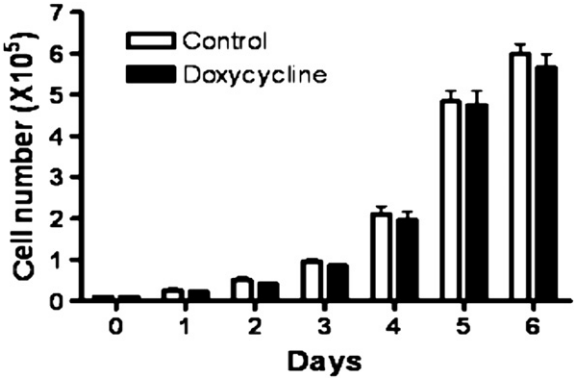

Figure 2. The proliferative rate was not significantly changed in doxycycline-induced SMN knockdown cells. 1×10^4 cells of the HeLa stable cell line were treated with 1 ug/mL doxycycline to induce survival motor neuron (SMN) protein knockdown. The cells without doxycycline treatment were used as control. The proliferative rates of cells were then analyzed by counting the number of cells. The cell numbers were counted everyday. This experiment was repeated three times. The error bars represent standard deviations calculated from three independent experiments. The slight decrease in doxycycline-induced SMN knockdown cells did not reach a statistically significant level by *T* test analysis ($p > 0.05$).

The survival rate of cells with reduced SMN protein level significantly decreased under oxidative stress

Because reduced SMN protein level alone may not be critical to cell viability, we further investigated other factors that may increase the cell death of SMN knockdown cells. Oxidative damage was reported to be the major cause for neuronal loss [18]. We then tested whether H_2O_2 increased the cell death of SMN knockdown cells. After induction by doxycycline, the SMN knockdown HeLa cells were treated with H_2O_2 from 0.01 mM to 6 mM for 24 hours (Fig. 3). This experiment was repeated three times. The results demonstrated that treatment with 0.01 mM, 0.25 mM, and 0.5 mM H_2O_2 did not cause significant difference in viabilities between cells with and without SMN knockdown. However, the survival rate of the cells with reduced SMN protein level significantly decreased with 0.75 mM and 1 mM H_2O_2 treatment. Moreover, almost all of the cells were dead when the concentration of H_2O_2 reached 6 mM (Fig. 3). These results indicate that certain concentrations of H_2O_2 increase the cell death of SMN knockdown cells.

Discussion

The fibroblasts from SMA patients are popular non-motor neurons cells used in SMN studies [19,20]. However, to collect fibroblasts is an invasive process for SMA patients. In addition, after the fibroblasts were cultured, the survival of

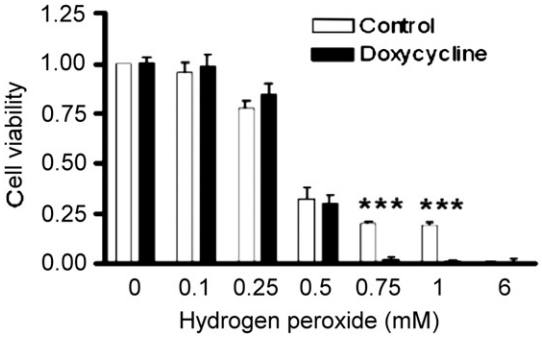

Figure 3. The survival rate significantly decreased in SMN knockdown cells with H_2O_2 treatment. Thousands of the HeLa stable cell line were cultured in 96-well plates and treated with doxycycline. The cells without doxycycline treatment were used as control. The cells were then treated with hydrogen peroxide from 0 mM to 6 mM for 24 hours. The cell survival assays were performed by CellTiter 96 Aqueous One Solution (Promega). Twenty microliters of CellTiter 96 Aqueous One Solution Reagent was added to each well, and the absorbance of 490 nm was measured. The values plotted in Y-axis represent the absorbent percentage of control without hydrogen peroxide treatment. The error bars represent standard deviations calculated from three independent experiments. Significant differences between doxycycline-treated and control cells are shown (*$p < 0.001$).

primary fibroblasts was passage limited. Therefore, to establish a cell model will be convenient for some of the preliminary data collection. SMA is characterized by reduced expression of SMN proteins. Gene knockdown is a powerful technique to study the role of SMN in SMA. However, most of the articles published to study SMA with RNA interference used transient expression systems, and it is difficult to perform long-term studies with these transient expression systems. The stable knockdown cells can be used for long-term studies. In this article, we successfully established a stably inducible SMN knockdown cell model system. Different SMN protein levels were induced by 0.5 ug/mL and 1 ug/mL doxycycline. This indicates that, with this cell model, the SMN protein level can be knocked down to certain ranges by different concentrations of doxycycline. The cells with different levels of SMN proteins will be useful for SMN functional studies.

The data in Fig. 2 showed that the proliferative rate of cells with reduced SMN proteins only slightly decreased in HeLa cells. However, Trüzsch et al. [12] showed that when *SMN* gene expression was knocked down in the differentiated P19 cells, it showed a dramatic increase in the percentage of apoptotic cells but not in the undifferentiated cells. It is, therefore, likely that SMN knockdown in different cell types results in different effects. HeLa cells may represent some non-motor neurons cell types. It is possible that the non-motor neurons cells of SMA patients are weaker than those of normal people especially under certain conditions. Our data in Figs. 2 and 3 show that reduced SMN protein level only slightly decreased the proliferative rate in HeLa cells. However, H_2O_2 dramatically increased the cell death of SMN knockdown cells. The cell viability of HeLa treated with H_2O_2 in our experiment is also similar to previous results with or without doxycycline [21–23]. These results demonstrated that some of the non-motor neurons cells in SMA patients may be normal in phenotype, but they are vulnerable under stresses, such as oxidative stress. Oxidative stress has been reported to be one of the predisposing factors in the pathogenesis of motor neuron diseases, including amyotrophic lateral sclerosis and Parkinson's disease [24–26]. Our results show that SMN plays an important role in non-neuronal cells under stressed conditions. It is still possible that motor neurons are also very sensitive under stresses, and the sensitivity to stresses of motor neurons is one of the major causes of the dysfunction in SMA patients. However, our results may be helpful to prevent some detrimental effects in SMA patients caused by defects in non-motor neurons. For example, it may be wise to advise SMA patients to avoid stresses, such as oxidative stress, in their daily health care.

Reactive oxygen species–generating agents, such as H_2O_2, menadione, and beta-lapachone, induce oxidative stress that inactivates the activity of SMN complex [27]. In addition, overexpressed SMN proteins protect cells against mutant superoxide dismutase 1 toxicity [28]. Superoxide dismutase 1 is an enzyme that is essential for scavenging of superoxide radicals. Thus, SMN may play a role in oxidative stress pathophysiology. Our data in Fig. 3 demonstrate that SMN participates in the cell death under H_2O_2 *in vivo*.

Overall, we successfully generated a cell model system with inducible SMN knockdown. With this cell model, we demonstrate that SMN participates in the cell death under oxidative stress. These findings may be helpful to prevent SMA patients from some detrimental effects. In addition to mechanistic studies, this cell model may be used for drug-screening assay to select the drugs that can improve the function of the cells with low levels of SMN protein. Our cell model may be a promising resource for studies on the mechanisms and clinical applications of SMA.

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