A Cell System for Phenotypic Screening of Modifiers of SMN2 Gene Expression and Function

Darrick K. Li1,2,9, Sarah Tisdale1,2,9, Jorge Espinoza-Derout1,2,9, Luciano Saieva1,2, Francesco Lotti1,2, Livio Pellizzoni1,2*

1Center for Motor Neuron Biology and Disease, Columbia University, New York, New York, United States of America, 2Department of Pathology and Cell Biology, Columbia University, New York, New York, United States of America

Abstract

Spinal muscular atrophy (SMA) is an inherited neurodegenerative disease caused by homozygous inactivation of the SMN1 gene and reduced levels of the survival motor neuron (SMN) protein. Since higher copy numbers of the nearly identical SMN2 gene reduce disease severity, to date most efforts to develop a therapy for SMA have focused on enhancing SMN expression. Identification of alternative therapeutic approaches has partly been hindered by limited knowledge of potential targets and the lack of cell-based screening assays that serve as readouts of SMN function. Here, we established a cell system in which proliferation of cultured mouse fibroblasts is dependent on functional SMN produced from the SMN2 gene. To do so, we introduced the entire human SMN2 gene into NIH3T3 cell lines in which regulated knockdown of endogenous mouse Smn severely decreases cell proliferation. We found that low SMN2 copy number has modest effects on the cell proliferation phenotype induced by Smn depletion, while high SMN2 copy number is strongly protective. Additionally, cell proliferation correlates with the level of SMN activity in small nuclear ribonucleoprotein assembly. Following miniaturization into a high-throughput format, our cell-based phenotypic assay accurately measures the beneficial effects of both pharmacological and genetic treatments leading to SMN upregulation. This cell model provides a novel platform for phenotypic screening of modifiers of SMN2 gene expression and function that act through multiple mechanisms, and a powerful new tool for studies of SMN biology and SMA therapeutic development.

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease characterized by loss of motor neurons in the anterior horn of the spinal cord and skeletal muscle atrophy [1]. SMA is caused by reduced levels of the survival motor neuron (SMN) protein, an evolutionarily conserved and ubiquitously expressed protein essential for viability [2,3]. SMN exists in a macromolecular complex with functions in the assembly of the small nuclear ribonucleoproteins (snRNPs) of the RNA splicing machinery and possibly other RNA-protein complexes [4–6]. In animal models of SMA, the disruption of snRNP biogenesis induced by SMN deficiency decreases snRNP levels [7–9] and causes splicing defects in genes that contribute to motor system dysfunction [10–12].

The human genome contains two genes that code for the SMN protein, SMN1 and SMN2, with multiple copies of SMN2 present [13]. SMA patients have homozygous loss or mutations of the SMN1 gene and retention of at least one copy of SMN2. Although the two SMN genes are nearly identical, a C to T transition in exon 7 of SMN2 results in the production of transcripts lacking exon 7 (SMNΔ7) with only a small proportion encoding full-length SMN [14–17]. SMN2 exon 7 skipping creates a destabilizing element responsible for the rapid degradation of the SMNΔ7 protein [18–20]. As a consequence, reduced levels of full-length SMN protein produced from the SMN2 gene, while sufficient to prevent embryonic lethality, are not able to fully compensate for the loss of SMN1 resulting in motor neuron disease.

There is a direct connection between SMN protein levels and SMA severity as higher SMN2 gene copy number correlates with milder forms of the disease in patients [21–23]. Thus, most efforts in developing SMA therapeutics have focused on methods to increase SMN protein levels. These include activation of the SMN2 promoter, enhancing inclusion of exon 7 in SMN2-derived transcripts, increasing the stability of SMN mRNA and protein, or restoring SMN expression through gene therapy [24–27]. In agreement with the viability of these approaches, injection of AAV vectors encoding full-length SMN in mouse models of SMA resulted in remarkable correction of lifespan and motor function [28–31]. Similarly, robust phenotypic benefit in SMA mice was accomplished by promoting SMN2 exon 7 inclusion using antisense oligonucleotides targeting intronic splicing silencers [32,33]. Small chemical compounds that increase SMN expression...
are also being investigated for SMA therapy. The use of histone deacetylase (HDAC) inhibitors has been shown to result in phenotypic improvement in SMA mouse models via SMN2 transcriptional upregulation [34,35]. Additional inducers of SMN expression have been identified in high-throughput chemical screens [36–40]. C5-substituted quinazolines [37], the most clinically advanced therapeutic candidates emerged from these screens, potentially inhibit the activity of the scavenger decapping enzyme DcpS [41] and improve survival and motor phenotype in SMA mice [42–44]. Although these candidate therapeutics are now entering clinical trials, SMA remains the most frequent genetic cause of infant mortality worldwide for which no effective treatment is currently available.

Ongoing efforts to address the high unmet clinical need in SMA would benefit from increased knowledge of basic SMN biology and disease mechanisms as well as identification of alternative therapeutic approaches. In addition to upregulation of SMN expression, strategies aiming to enhance SMN function or to correct downstream effects of SMN deficiency might provide new avenues for SMA therapy. Accordingly, there is evidence that SMN function is regulated both in vivo and in vitro [45,46] and that targeting downstream defects induced by SMN deficiency without increasing SMN levels can be beneficial in animal models of SMA [10,11,47–50]. However, progress towards the development of alternative therapeutic strategies for SMA has generally been hindered by lack of suitable targets as well as screening platforms that supply readouts of SMN function and downstream SMN-dependent events. In an effort to address this shortcoming, we developed a novel cell-based system for phenotypic screening of chemical or genetic modifiers that may increase SMN expression and function through multiple mechanisms of action. To do so, we have taken advantage of a previously characterized mouse NIH3T3 cell line in which regulated knockdown of endogenous mouse Smn triggers a severe cell proliferation defect [11], providing a direct phenotypic readout of SMN function. We introduced the human SMN2 gene into this cell line, generating a system where cell proliferation is sensitive to changes in functional SMN levels produced from SMN2. We further miniaturized and validated a cell-based phenotypic assay in 96-well format amenable to high-throughput chemical and genetic screens. Our cell model system provides a novel tool for the discovery of cellular factors and genetic networks that control SMN biology in mammalian cells, which will not only increase our knowledge of the basic biology of SMN but also contribute to SMA therapeutic development.

Results

Development of Human SMN2-containing NIH3T3 Cell Lines with Regulated Knockdown of Endogenous Smn

We recently established and characterized an NIH3T3-SmnRNAi cell line with drug-inducible, RNAi-mediated knockdown of endogenous mouse Smn [11,12]. In these cells, addition of doxycycline causes Smn depletion and subsequent, severe cell proliferation defects that can be corrected by transgenic expression of RNAi-resistant human SMN [11]. We sought to modify this model system so that cell proliferation would be dependent on SMN levels produced by the human SMN2 gene. To do so, the 33.5 kb BamH I fragment corresponding to the genomic region encompassing the SMN2 gene (Figure 1A), previously used to generate SMA mice [51], was cloned into a cosmid vector containing a neomycin selection cassette under the control of the SV40 promoter and the resulting construct was transfected into NIH3T3-SmnRNAi cells. Several neomycin-resistant stable cell lines were isolated through antibiotic selection and cloning by limiting dilution in 96-well plates. Here, we describe the characterization of two representative NIH3T3-SMN2/SmnRNAi cell lines with either low or high SMN2 copy number. Genomic DNA from these NIH3T3 cell lines was isolated and the relative SMN2 gene copy number was determined by quantitative PCR with human SMN-specific primers. NIH3T3-SMN2high/SmnRNAi cells contained more than tenfold the number of SMN2 copies present in NIH3T3-SMN2low/SmnRNAi cells (Figure 1B). Consistent with this, RT-qPCR analysis with human-specific SMN primers showed that in the absence of doxycycline NIH3T3-SMN2high/SmnRNAi cells expressed approximately ten times the amount of total SMN2 mRNA compared to NIH3T3-SMN2low/SmnRNAi cells (Figure 1C). Importantly, radioautographic RT-PCR analysis revealed the expected pattern of exon 7 splicing regulation of the SMN2 gene in both cell lines (Figure 1D). Irrespective of the marked difference in the overall levels SMN2 expression, the majority of SMN2 transcripts lacked exon 7 (SMNΔ7) and only ~10% were full-length SMN mRNA.

Next, we investigated the expression and subcellular localization of the human SMN protein in NIH3T3-SMN2/SmnRNAi cells. In agreement with the SMN2 gene copy number and mRNA expression levels, Western blot analysis with an antibody that specifically detects human SMN revealed that in the absence of doxycycline NIH3T3-SMN2high/SmnRNAi cells express approximately tenfold more SMN than NIH3T3-SMN2low/SmnRNAi cells (Figure 2). Human SMN protein was not detected in either wild-type or NIH3T3-SmnrRNAi cells that do not contain the SMN2 gene, confirming the specificity of the antibodies. We then carried out immunofluorescence experiments with a human SMN-specific antibody (anti-hSMN) or an antibody that recognizes both mouse and human SMN proteins (anti-SMN) in NIH3T3 cell lines cultured without doxycycline. Analysis in NIH3T3-SmnrRNAi cells showed no staining with anti-hSMN but strong cytoplasmic and weak nuclear staining with anti-SMN (Figure 3A and 3D). As expected, staining with anti-hSMN was weak in NIH3T3-SMN2low/SmnRNAi cells (Figure 3B) and strong in NIH3T3-SMN2high/SmnRNAi cells (Figure 3C), and revealed a similar subcellular distribution to that of endogenous mouse Smn. Noticeably, SMN localization in nuclear foci known as Gems [52] while rare in wild-type NIH3T3 cells was frequently observed in NIH3T3-SMN2high/SmnRNAi cells, possibly due to higher SMN levels.

Lastly, we investigated whether the capacity for doxycycline-inducible, RNAi-mediated knockdown of endogenous Smn characteristic of parental NIH3T3-SmnRNAi cells was retained in the newly established NIH3T3-SMN2/SmnRNAi cell lines. We first analyzed the effects of doxycycline on the expression levels of mouse Smn mRNA by RT-qPCR. As expected [11], doxycycline-treated NIH3T3-SmnrRNAi cells had strongly decreased levels of Smn mRNA compared to untreated cells and doxycycline had no effects in wild-type NIH3T3 cells (Figure 4A). Doxycycline caused a reduction in Smn mRNA levels in both NIH3T3-SMN2low/SmnRNAi and NIH3T3-SMN2high/SmnRNAi cells similar to that in NIH3T3-SmnrRNAi cells, demonstrating that these cells preserved the ability for inducible knockdown of Smn mRNA. Consistent with the shRNA specific targeting of mouse Smn mRNA, human SMN2 mRNA levels were unaffected by RNAi induction with doxycycline in NIH3T3-SMN2/SmnRNAi cells (Figure 4B). We then carried out Western blot analysis to determine the levels of SMN protein expression following knockdown of endogenous Smn. In agreement with Smn mRNA levels and our previous studies [11,12], doxycycline treatment of NIH3T3-SmnrRNAi cells for 7 days strongly reduced SMN protein levels at approximately
10% of normal, but had no effect in wild-type NIH3T3 cells (Figure 4C). Importantly, the amounts of SMN in doxycycline-treated NIH3T3−SMN2low/SmnRNAi (20%) and NIH3T3−SMN2high/SmnRNAi cells (80%) relative to untreated cells were consistent with the levels of human SMN mRNA expressed from the SMN2 gene. Thus, we developed NIH3T3 cell lines with regulated knockdown of endogenous Smn that express either low or high levels of human SMN from the SMN2 gene.

### Cell Proliferation Correlates with SMN2 Gene Expression and Function in Smn-deficient NIH3T3-SMN2/SmnRNAi Cells

SMN deficiency elicits a severe cell proliferation phenotype in NIH3T3 cells [11]. Smn-deficient NIH3T3−SmnRNAi cells display decreased proliferation after 3 days of doxycycline treatment and become growth arrested at day 5, entering a quiescent proliferative state that could last for many days without significant cell death [11]. While doxycycline has no effect in wild-type NIH3T3 cells, transgenic expression of human SMN is able to correct cell proliferation defects in Smn-deficient NIH3T3 cells [11], indicating that the effects are specifically due to SMN depletion. We therefore investigated the effect of SMN2 gene expression on cell proliferation in NIH3T3−SMN2/SmnRNAi cell lines cultured in the presence or absence of doxycycline for 7 days (Figure 5A). SMN deficiency resulted into a twenty-fold difference in the number of NIH3T3−SmnRNAi cells relative to wild-type NIH3T3 cells, in

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**Figure 1. Development of SMN2-containing NIH3T3 cell lines with inducible knockdown of endogenous Smn.** (A) Schematic representation of the exon-intron structure of the human SMN2 gene used to establish NIH3T3−SMN2/SmnRNAi cell lines (introns not drawn to scale). (B) Analysis of SMN2 gene copy number in the indicated NIH3T3 cell lines by qPCR. Data were normalized to the Gapdh gene and expressed relative to the NIH3T3−SMN2low/SmnRNAi cell line. Data are represented as mean and SEM (n = 3; ** = p < 0.01; t-test). (C) Analysis of total SMN2 mRNA levels in the indicated NIH3T3 cell lines by RT-qPCR. Data were normalized to Gapdh mRNA and expressed relative to the NIH3T3−SMN2low/SmnRNAi cell line. Data are represented as mean and SEM (n = 3; *** = p < 0.001, t-test). (D) Analysis of SMN2 exon 7 splicing in the indicated NIH3T3 cell lines by radioactive RT-PCR. The levels of exon 7 inclusion are shown at the bottom.

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**Figure 2. Analysis of human SMN protein levels in NIH3T3−SMN2/SmnRNAi cell lines.** Western blot of equal amounts of proteins from wild-type (Control), NIH3T3−SmnRNAi, NIH3T3−SMN2low/SmnRNAi and NIH3T3−SMN2high/SmnRNAi cell lines with monoclonal antibodies against the indicated proteins. Monoclonal antibodies specific to human SMN (hSMN) or both mouse and human SMN (SMN) were used. A two-fold serial dilution of the extract from NIH3T3−SMN2high/SmnRNAi cells is shown on the right.

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which doxycycline had no effect on proliferation. Consistent with the expression of low levels of human SMN from the \textit{SMN2} gene, NIH3T3-SMN2\textsubscript{low}/\textit{SmnRNAi} cells exhibited a strong cell proliferation defect following knockdown of endogenous Smn, but this effect was significantly less severe than that in NIH3T3-Smn\textit{RNAi} cells. In particular, despite very slow cell proliferation, Smn-deficient NIH3T3-SMN2\textsubscript{low}/\textit{SmnRNAi} cells unlike NIH3T3-Smn\textit{RNAi} cells did not stop growing even after prolonged culture in the presence of doxycycline. Increased levels of human SMN expression from the \textit{SMN2} gene in NIH3T3-SMN2\textsubscript{high}/\textit{SmnRNAi} cells resulted in a robust albeit incomplete correction of the cell proliferation phenotype triggered by Smn deficiency, with NIH3T3-SMN2\textsubscript{high}/\textit{SmnRNAi} cells on average cycling 10% more slowly than wild-type NIH3T3 cells in the presence of doxycycline. These results indicated that proliferation of Smn-deficient NIH3T3-SMN2/\textit{SmnRNAi} cells is proportional to the levels of human SMN expressed from the \textit{SMN2} gene.

Next, we investigated SMN function and its correlation with proliferation in NIH3T3-SMN2/\textit{SmnRNAi} cell lines. The only molecularly defined activity of SMN is in the assembly of the Sm core on spliceosomal snRNAs [53,54] and the degree of reduction in snRNP assembly correlates with disease severity in mouse models of SMA [7]. We previously showed that snRNP assembly is severely decreased in Smn-deficient NIH3T3-Smn\textit{RNAi} cells and restored by expression of human SMN, while doxycycline has no effect [11]. We carried out \textit{in vitro} snRNP assembly experiments using radioactive U1 snRNA and extracts from NIH3T3 cell lines cultured with or without doxycycline followed by immunoprecipitation with anti-SmB antibodies to monitor Sm core formation \textit{in vitro}. These experiments showed that the SMN-dependent snRNP assembly defects are slightly (two-fold) less severe in NIH3T3-SMN2\textsubscript{low}/\textit{SmnRNAi} cells compared to NIH3T3-Smn\textit{RNAi} cells that do not contain the \textit{SMN2} gene and significantly rescued in NIH3T3-SMN2\textsubscript{high}/\textit{SmnRNAi} cells (Figure 5B). Overall, these data demonstrated that SMN expression from the \textit{SMN2} gene correlates well with snRNP assembly activity and cell proliferation in our model system.

**Development of a Cell-based Phenotypic Assay for SMN-dependent Cell Proliferation in NIH3T3 Cells**

We sought to develop a cell-based assay for high-throughput chemical and genetic screens of modifiers of SMN expression and function that uses reduced proliferation as a robust phenotypic readout of SMN deficiency in NIH3T3 cells. First, we developed an automated, imaging-based approach to determine cell number in 96-well format as a measure of SMN-dependent cell proliferation in NIH3T3 cells. Serial dilutions of NIH3T3-SMN2\textsubscript{low}/\textit{SmnRNAi} cells were seeded in a 96-well optical plate, followed by fixation and nuclear staining with Hoechst 4 hours later. Direct determination of cell number was then carried out by whole well imaging with an IN Cell Analyzer. These experiments demonstrated a linear relationship between the number of cells plated and the optical readout of Hoechst-stained nuclei (Figure 6A). Thus, in addition to being rapid and cost-effective, this methodology showed remarkable linearity over a wide range of cell number.

Next, we investigated whether SMN-dependent effects on cell proliferation in NIH3T3 cells could be assessed using a 96-well format and the above readout. To do so, NIH3T3-Smn\textit{RNAi} and NIH3T3-SMN2\textsubscript{low}/\textit{SmnRNAi} cells were cultured with and without doxycycline for 5 days prior to seeding 200 cells into 6 replicate wells of a 96-well plate, with doxycycline treatment continuing throughout the course of the experiment. Cell number was determined at 4 hours (time zero) as well as 4 and 5 days post-plating using Hoechst staining followed by imaging with the IN Cell Analyzer. Figure 6B shows representative whole well images of normal and SMN-deficient NIH3T3-SMN2\textsubscript{low}/\textit{SmnRNAi} cells at 5 days post-plating in 96-well plates. These experiments

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Figure 3. Subcellular localization of the human SMN protein in NIH3T3-SMN2/\textit{SmnRNAi} cell lines. Indirect immunofluorescence and confocal microscopy analysis of NIH3T3-Smn\textit{RNAi} (A and D), NIH3T3-SMN2\textsubscript{low}/\textit{SmnRNAi} (B and E) and NIH3T3-SMN2\textsubscript{high}/\textit{SmnRNAi} (C and F) cell lines using monoclonal antibodies specific to human SMN (hSMN, panels A–C) or both mouse and human SMN (SMN, panels D–F). Scale bar, 10 \textmu m. doi:10.1371/journal.pone.0071965.g003
revealed a similar time-dependent increase in the number of untreated NIH3T3-SmnRNAi and NIH3T3-SMN2low/SmnRNAi cells with normal levels of SMN and the time zero analysis confirmed the presence of equal numbers of cells at plating (Figure 6C-D). Consistent with the growth arrest phenotype at the time of plating, the number of doxycycline-treated NIH3T3-SmnRNAi cells did not change over time (Figure 6C). In contrast, doxycycline-treated NIH3T3-SMN2low/SmnRNAi showed a mod-

Figure 4. Inducible knockdown of endogenous mouse Smn in NIH3T3-SMN2/SmnRNAi cell lines. (A) RT-qPCR analysis of mouse Smn mRNA levels in wild-type (Control), NIH3T3-SmnRNAi, NIH3T3-SMN2low/SmnRNAi and NIH3T3-SMN2high/SmnRNAi cell lines cultured with and without Dox for 7 days. Data in Dox-treated cells were normalized to those in untreated cells and represented as mean and SEM (n = 3; *** = p<0.001; t-test). (B) RT-qPCR analysis of human SMN2 mRNA levels in NIH3T3-SMN2low/SmnRNAi and NIH3T3-SMN2high/SmnRNAi cell lines cultured with and without Dox for 7 days. Data in Dox-treated cells were normalized to those in untreated cells and represented as mean and SEM. (C) Western blot analysis of equal amounts of proteins from wild-type (Control), NIH3T3-SmnRNAi, NIH3T3-SMN2low/SmnRNAi and NIH3T3-SMN2high/SmnRNAi cell lines cultured with and without Dox for 7 days using monoclonal antibodies against the indicated proteins. For each cell line, SMN levels in Dox-treated relative to untreated cells are shown at the bottom. doi:10.1371/journal.pone.0071965.g004
est time-dependent increase in cell number (Figure 6D), consistent
with both a much slower rate of proliferation relative to untreated
cells and the differential severity of the phenotype triggered by
Smn deficiency in NIH3T3-SMN2low/SmnRNAi compared to
NIH3T3-SmnRNAi cells (see also Figure 5). The largest difference
in cell number between normal and Smn-deficient cells was
observed at 5 days post-plating for both NIH3T3-SmnRNAi (40-
fold) and NIH3T3-SMN2low/SmnRNAi (~5-fold) cells. Thus, we
established a cell-based phenotypic assay in 96-well format that
accurately measures SMN-dependent effects on proliferation of
NIH3T3 cells.

Genetic and Pharmacological Modulation of SMN-
dependent Cell Proliferation in NIH3T3 Cells

Next we determined whether the cell proliferation defects
caused by SMN deficiency in NIH3T3 cells could be modulated
by genetic and chemical approaches. First, we investigated the
capacity for the system to respond to genetic approaches by
assessing the ability of lentiviral-mediated human SMN expression
to correct the cell proliferation phenotype in Smn-deficient
NIH3T3 cells using our 96-well format assay. Both NIH3T3-
SmnRNAi and NIH3T3-SMN2low/SmnRNAi cells in which Smn
deficiency was induced by preincubation with doxycycline for 5
days were plated in 6 replicate wells of 96-well plates in the
presence of doxycycline as well as increasing amounts of lentivirus
expressing human SMN driven by the CMV promoter. The
number of vehicle- and lentivirus-treated NIH3T3 cells was
determined 5 days later using Hoechst nuclear staining and IN
Cell Analyzer imaging. These experiments revealed that human
SMN expression promoted cell proliferation of Smn-deficient
NIH3T3 cells in a dose-dependent manner with a maximum
increase in cell number of 3.5-fold for NIH3T3-SmnRNAi cells and
nearly 3-fold for NIH3T3-SMN2low/SmnRNAi cells compared to
their corresponding vehicle-treated controls (Figure 7). With the
amount of lentivirus that most effectively promotes cell prolifer-
ation, the level of human SMN expressed is equivalent to
endogenous mouse Smn in wild-type NIH3T3 cells (data not
shown). Furthermore, lentiviral-mediated expression of GFP under
the same conditions had no effect on the proliferation of Smn-
deficient NIH3T3 cells (data not shown), confirming the specificity
for the effects of SMN restoration.

We also tested whether SMN-dependent cell proliferation could
be modulated by treatment with small chemical compounds. We
used the histone deacetylase inhibitor valproic acid (VPA), which
was previously demonstrated to increase SMN levels in human
SMA fibroblasts [55,56], leading to improved motor function and
survival in a mouse model of SMA [57]. We performed dose-
response analysis of VPA effects on cell proliferation of doxycy-
cline-treated NIH3T3-SMN2low/SmnRNAi and NIH3T3-
SmnRNAi cells using the same conditions employed in lentiviral
transduction experiments. We found that VPA treatment resulted
in dose-dependent stimulation of cell proliferation in Smn-

Figure 5. Effect of human SMN2 expression on proliferation and snRNP assembly in Smn-deficient NIH3T3 cell lines. (A) Analysis of
cell proliferation in wild-type (Control), NIH3T3-SmnRNAi, NIH3T3-SMN2low/SmnRNAi, and NIH3T3-SMN2high/SmnRNAi cell lines cultured with and without
doxycycline for 7 days. For each cell line, the cell number ratio of Dox-treated cells versus untreated cells is expressed relative to that of wild-type
cells, which is set to 1. Data are represented as mean and SEM (n ≥ 3; *** = p < 0.001; one-way ANOVA). (B) Analysis of U1 snRNP assembly in NIH3T3
cell lines. In vitro snRNP assembly experiments were carried out with radioactive U1 snRNA and extracts from wild-type NIH3T3 (Control), NIH3T3-
SmnRNAi, NIH3T3-SMN2low/SmnRNAi and SMN2high/SmnRNAi cells cultured with and without doxycycline for 7 days. For each cell line, the amounts of immunoprecipitated U1 snRNA were quantified and the RNA ratio in Dox-treated cells versus untreated cells is expressed relative to that of wild-type
cells, which is set to 1. Data are represented as mean and SEM (n ≥ 3; * = p < 0.05; ** = p < 0.01; *** = p < 0.001; one-way ANOVA).
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deficient NIH3T3-SMN2low/SmnRNAi cells compared to vehicle-treated controls (Figure 8A). There was a maximum 2.5-fold increase in cell number at 1 mM, while higher drug concentrations appeared toxic. Importantly, VPA treatment had no effect on the proliferation of Smn-deficient NIH3T3-SmnRNAi cells lacking the SMN2 gene (Figure 8A). The specificity of the effects for NIH3T3-SMN2low/SmnRNAi cell lines containing the SMN2 gene was consistent with the predicted mechanism of action for VPA in increasing SMN2 gene expression. To determine whether this was indeed the case, we compared SMN mRNA and protein levels in Smn-deficient NIH3T3-SMN2low/SmnRNAi and NIH3T3-SmnRNAi cells treated with 1 mM VPA relative to vehicle-treated controls. RT-qPCR experiments showed that VPA treatment did not change the low level of Smn mRNA in either NIH3T3-SMN2low/SmnRNAi or NIH3T3-SmnRNAi cells (Figure 8B–C), indicating that it did not interfere with the doxycycline-dependent RNAi knockdown of endogenous mouse Smn. In contrast, VPA treatment resulted in a 1.5-fold increase in total SMN mRNA produced from the SMN2 gene in NIH3T3-SMN2low/SmnRNAi cells compared to vehicle-treated cells (Figure 8C). A similar increase was also found for full-length SMN mRNA (data not shown). In agreement with the observed changes in mRNA levels, Western blot analysis showed that the low Smn protein levels were unaffected by VPA in Smn-deficient NIH3T3-SmnRNAi cells (Figure 8D), while VPA treatment increased SMN protein levels (1.7-fold, p < 0.01) in NIH3T3-SMN2low/SmnRNAi cells compared

Figure 6. An SMN-dependent cell proliferation assay in 96-well format. (A) Automated determination of NIH3T3 cell number in 96-well format. Two-fold serial dilutions of NIH3T3-SMN2low/SmnRNAi cells were plated in eight replicate wells of a 96-well plate. Following fixation and Hoechst staining 4 hours later, cell number was determined by imaging whole wells with an IN Cell Analyzer 2000. (B) Representative IN Cell Analyzer images of NIH3T3-SmnRNAi cells cultured with or without Dox in a 96-well format. (C–D) Analysis of SMN-dependent cell proliferation in NIH3T3-SmnRNAi (C) and NIH3T3-SMN2low/SmnRNAi (D) cells using the 96-well format assay. NIH3T3 cells were cultured for 5 days with or without Dox, and then seeded in six replicate wells of a 96-well plate. Dox-treatment was continued throughout the experiment. Cell number was determined at 4 hours (T0), 4 days and 5 days post-plating. Data are represented as mean and SEM (n = 6; *** = p < 0.001; two-way ANOVA).

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to vehicle-treated cells (Figure 8E). These results were consistent with the beneficial effects of VPA on proliferation of NIH3T3-SMN2low/SmnRNAi cells being the consequence of SMN upregulation. The observation that moderate SMN induction with VPA elicits an effect on cell proliferation of NIH3T3-SMN2low/SmnRNAi cells similar to that of complete, lentiviral-mediated SMN restoration can be explained by differences in the timing of SMN expression. Characteristic of the lentiviral system, there is a lag of approximately one day between viral transduction and the onset of transgenic SMN expression, which delays the biological effect of SMN restoration. In contrast, small molecules such as VPA can readily engage their cellular targets and act more quickly. It is therefore possible, if not likely that some chemical compounds could have a more robust effect than we observe with the SMN lentivirus in our cell proliferation assay.

Collectively, these experiments provided proof of concept that cell proliferation defects induced by Smn deficiency could be improved by genetic or pharmacological upregulation of SMN in NIH3T3-SmnRNAi cells and thus used as a phenotypic readout in future screens for modifiers of SMN2 gene expression or function.

**Discussion**

SMA is a devastating neurological disease for which no effective treatment is currently available. Progress in the discovery of new SMA therapeutic approaches has been hampered, at least in part, by the relatively limited knowledge of targets and the lack of assays in high-throughput format that provide a functional readout of SMN activity. To facilitate the identification of novel targets and therapeutics that act not only on SMN expression but also on SMN function and downstream events induced by SMN deficiency, we developed a cell-based system that uses cell proliferation defects triggered by SMN deficiency in cultured mammalian cells as phenotypic readout of the functional levels of SMN produced from the SMN2 gene. Following miniaturization of this cell-based assay for use in high-throughput format, we provide proof of principle for the application of this novel platform for unbiased, genetic and chemical screens that aim to identify and characterize modifiers of SMN expression and function that act through any one of multiple possible mechanisms of action. Thus, our work establishes a powerful new discovery tool for the study of SMN biology and possibly the development of novel approaches for SMA therapy.

SMN is an essential gene that is required for cell growth and viability from yeast to mammals [11,58–62]. The model system we developed is based on a mouse NIH3T3 fibroblast cell line (NIH3T3-SmnRNAi) in which regulated knockdown of endogenous mouse Smn triggers a severe cell proliferation defect [11], providing a direct phenotypic readout of SMN function. The rationale of the work was to adapt this system for purposes of therapeutic and biological discovery in SMA research by introducing the entire human SMN2 gene into NIH3T3-SmnRNAi cells, thereby making cell proliferation dependent on the levels of functional SMN produced from the SMN2 gene. We successfully isolated NIH3T3 cell lines with either low or high SMN2 copy numbers and demonstrated that cell proliferation is proportional to the levels of human SMN expression following RNAi depletion of endogenous Smn. Consistent with the phenotypic effects of varying SMN2 copy numbers in mice lacking the Smn alleles [51,63], we found that low SMN2 copy number modestly improves the proliferation phenotype of Smn-deficient NIH3T3 cells, while

**Figure 7. Genetic modulation of SMN-dependent proliferation in NIH3T3 cells.** Dose-response analysis of lentiviral-mediated human SMN expression on cell proliferation of SMN-deficient NIH3T3-SrnRNAi and NIH3T3-SMN2low/SmnRNAi cells. In these experiments, NIH3T3 cells were cultured for 5 days with Dox prior to seeding in six replicate wells of a 96-well plate in the presence of Dox. Vehicle or increasing amounts of SMN-expressing lentivirus were added 4 hours later. For each group and treatment, cell number was determined at 5 days post-plating and normalized to that of the corresponding vehicle-treated cells. Data are represented as mean and SEM (n = 6; * = p < 0.05; ** = p < 0.01; *** = p < 0.001; one-way ANOVA).

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A Model for Functional Analysis of SMN Modifiers

A

![Graph showing normalized cell number against VPA concentration]

B

![Graph showing mRNA levels with and without VPA]

C

![Graph showing mRNA levels with and without VPA for SMN2 and SmnRNAi]

D

![Western blot analysis showing SMN and tubulin levels with and without VPA for SmnRNAi]

E

![Western blot analysis showing SMN and tubulin levels with and without VPA for SMN2 and SmnRNAi]

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high SMN copy number is remarkably protective. In animal models of SMA, snRNP assembly defects correlate with disease severity [7] and restoration of snRNP levels coincides with phenotypic correction [8,64]. Similarly, we show a direct correlation between the degree of SMN-dependent snRNP assembly impairment and the severity of the cell proliferation phenotype in our model system. Moreover, functional analysis of several SMN point mutations associated with SMA revealed that their ability to support snRNP assembly and correct the proliferation defects in Smn-deficient NIH3T3 fibroblasts is proportional to their potency in rescuing motor neuron phenotypes in animal models and inversely correlated with clinical severity in patients (L. Pelizzoni, C. Beattie and A. Burghes, unpublished results). Lastly, reduced cell proliferation induced by SMN deficiency in select brain regions has been associated with impaired perinatal brain development in severe SMA mice [65].

Altogether, this evidence supports the conclusion that the effect of SMN deficiency on cell proliferation in this system mirrors at least some of the mechanisms at play in the disease.

As the copy number of the nearly identical SMN2 gene correlates with disease severity [21–23], SMA therapeutic development has primarily focused on enhancing the expression of full-length SMN from the SMN2 gene or through gene therapy [24–27]. To date, cell-based screens for SMA therapeutic discovery have employed reporter assays that monitor increased SMN2 transcription and exon 7 splicing [36–39]. Immunodetection and imaging methodologies were also used to screen for small molecules that upregulate SMN protein levels in human SMA fibroblasts [40]. However, cell-based phenotypic assays that measure increased SMN expression and function from the SMN2 gene and are amenable to high-throughput screening have not been developed. Thus, our cell system has a number of distinct benefits, which complement and significantly advance currently available tools for SMA translational research, allowing the opportunity to identify cellular factors and genetic networks linked to SMN biology in a comprehensive and mechanistically unbiased manner. First, the presence of the entire SMN2 gene allows for the identification of potential modifiers acting at any level of SMN gene expression from transcription to protein turnover. Second, our platform is able to capture the possible beneficial effects of modifiers that act not only on SMN expression but also on its activity as well as downstream SMN-dependent pathways. Lastly, comparison of the effects of candidate chemical and genetic modifiers on the proliferation of SMN-deficient NIH3T3-SmnRNAi and NIH3T3-SMN2/SmnRNAi cell lines will provide the opportunity for effective hit deconvolution and rapid identification of modifiers acting through SMN2-dependent and -independent mechanisms for priority ranking and early mechanistic insights.

The robust cell proliferation phenotype of NIH3T3-SMN2/SmnRNAi cells combined with the development of an assay in 96-well format provides the foundation for the applicability of our platform to high-throughput screening. Additionally, direct measure of NIH3T3 cell number by a single-step method comprising nuclear Hoescht staining followed by automated imaging is a rapid, cost-effective and accurate method for assessing SMN-dependent cell proliferation in the 96-well format. In support of the conclusion that our phenotypic platform is suitable for screening modifiers of SMN biology, we provide proof-of-concept that it can respond to either genetic or pharmacological intervention. We show that lentiviral-mediated SMN transduction promotes proliferation of both NIH3T3-SMN2/SmnRNAi and NIH3T3-SMN2/SmnRNAi cells in a dose-dependent and SMN2-independent manner, highlighting the specificity and reversibility of the phenotype induced by Smn deficiency. Conversely, consistent with SMN2 gene upregulation and the results of previous studies [55,56], we show that the beneficial effects of treatment with the HDAC inhibitor VPA on cell proliferation are restricted to Smn-deficient NIH3T3 cells containing SMN2. Together, these features support the viability of our system for use in high-throughput chemical and genetic screening projects for SMA discovery.

In conclusion, the cell system for phenotypic screening we developed provides a powerful new tool for identifying and validating modifiers of SMN expression and function, which promise to yield not only critical insights into our understanding of the basic biology of SMA but also new therapeutic avenues for the treatment of SMA.

Materials and Methods

**NIH3T3 Cell Lines and Tissue Culture**

The NIH3T3 cell line with inducible knockdown of endogenous mouse Smn (NIH3T3-SmnRNAi) used in this study has been described previously [11,12]. To generate human SMN2-containing NIH3T3 cell lines with regulated Smn knockdown, the 35.5 kb BamHI fragment corresponding to the human genomic region encompassing the SMN2 gene, previously used to generate SMA transgenic mice [31], was excised from a BAC construct (a gift from Dr. Arthur Burghes) and cloned into the SuperCos1 cosmid vector (Stratagene). The resulting vector was transfected into NIH3T3-SmnRNAi cells and stable NIH3T3-SMN2/SmnRNAi cells were isolated through antibiotic selection with G418 (0.5 mg/ml). Clonal cell lines were obtained by limiting dilution in 96-well plates.

NIH3T3 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) with high glucose (Gibco) containing 10% fetal bovine serum (HyClone), 2 mM glutamine (Gibco), and 0.1 mg/ml gentamicin (Gibco). Smn RNAi was induced by addition of doxycycline (Fisher) to the growth medium at a final concentration of 100 ng/ml as previously described [11,12].

Lentiviral constructs expressing GFP and SMN driven by the CMV promoter were generated by standard cloning techniques using the pRRLSIN.cPPT.PGK-GFP.WPRE vector (Addgene plasmid 12252) as a backbone [66,67]. For lentivirus production, viral stocks pseudotyped with the vesicular stomatitis G protein (VSV-G) were prepared by transient co-transfection of 293 T cells...
as previously described [11]. Lentiviral particles in supernatants were harvested, concentrated by ultracentrifugation for 2 hours and 30 minutes at 19,500 rpm in a SW28 rotor (Beckman), and titered using the Lenti-X<sup>TM</sup> qRT-PCR Titration Kit (Clontech).

**DNA and RNA Analysis**

For genomic DNA analysis, cells were washed twice in ice cold PBS and harvested into 500 µl of PK buffer (10 mM EDTA, 100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2% SDS) containing 1 mg/ml protease K followed by overnight incubation at 50°C with shaking at 800 rpm. After addition of 500 µl of isopropanol and further incubation for 2 hours at room temperature, genomic DNA was pulled out of solution and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 7.5).

Total RNA from NIH3T3 cells was isolated using Trizol reagent (Invitrogen) followed by digestion with DNase I (Ambion). RNA (1 µg) was reverse transcribed using RevertAid First Strand cDNA Kit (Fermentas). For analysis of SNM2 exon 7 splicing, semi-quantitative RT-qPCR reactions were carried out in the presence of a forward primer 5’ end-labeled using T4 polynucleotide kinase (New England BioLabs) and [32P]-ATP (Perkin Elmer) as previously described [12]. PCR products were amplified using AmpliTaq Gold DNA polymerase (Roche), resolved on an 8% polyacrylamide-8 M urea gel, and subjected to autoradiography. The linear range of PCR amplification was determined independently for each experiment. Quantification of radiolabeled PCR products was carried out using a Typhoon PhosphorImager (Molecular Dynamics). For quantitative RT-qPCR experiments, triplicate reactions were carried out using Power SYBR Green PCR master mix (Applied Biosystems) in a Realplex<sup>4</sup> Mastercycler. For quantitative RT-qPCR experiments, triplicate reactions were carried out using Power SYBR Green PCR master mix (Applied Biosystems) in a Realplex<sup>4</sup> Mastercycler. RT-qPCR data were normalized to Gapdh mRNA. The primers utilized in this study were previously reported [12].

**Antibodies**

The following antibodies were used in this study: anti-SMN clone 8 (BD Transduction Laboratories), anti-SMN 7F3 [68], anti-SmB 18F6 [69], anti-human SMN (a gift from Dr. Adrian Krainer), and anti-Tubulin DM 1A (Sigma).

**Western Blot Analysis**

Analysis was performed as described previously [12]. In short, proteins were fractionated by SDS/PAGE on 12% polyacrylamide gels and transferred onto a Trans-Blot transfer medium nitrocellulose membrane (Bio-Rad) using 1X Tris-glycine buffer (Bio-Rad) containing 20% methanol. The blots were blocked in 5% nonfat dry milk in PBS containing 0.1% Tween 20 for 1 h at room temperature. Membranes were washed 3 times for 10 minutes with PBS containing 0.1% Tween 20 at room temperature. Incubation with secondary antibodies conjugated to horseradish peroxidase was performed in PBS containing 0.1% Tween 20 for 1 h at room temperature. Chemiluminescence was carried out using a Supersignal West Pico Chemiluminescent substrate according to the supplier’s recommendations (Thermo Scientific). SMN protein levels were quantified after normalization to Tubulin using ImageJ.

**Immunofluorescence Analysis**

NIH3T3 cells were grown on glass coverslips in 24-well plates. Cells were washed once with PBS and then fixed in 4% paraformaldehyde-PBS for 15 minutes at room temperature. Following fixation, cells were permeabilized in 0.5% Triton-X in PBS for 5 minutes at room temperature. Blocking and both primary and secondary antibody incubations were performed with 3% BSA in PBS. Images were collected with a SP5 confocal microscope (Leica).

**Cell Proliferation Assay in 96-well Format**

In vitro snRNP assembly experiments were performed using radioactive U1 snRNA and NIH3T3 cell extracts (25 µg) followed by immunoprecipitation with anti-Smn antibodies, electrophoresis on denaturing polyacrylamide gels and autoradiography as previously described [7,11]. Quantification of immunoprecipitated U1 snRNA was carried out using a Typhoon PhosphorImager (Molecular Dynamics).

**Statistical Analysis**

Statistical analysis was carried out with the Prism 5 (GraphPad) software using two-tailed unpaired Student’s t-test and one-way or two-way ANOVA followed by the Bonferroni post-hoc test as indicated. Data are represented as mean plus SEM and P values are indicated as follows: * = p<0.05; ** = p<0.01; *** = p<0.001.

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**Author Contributions**

Conceived and designed the experiments: DL ST JD LP. Performed the experiments: DL ST JD. Analyzed the data: DL ST JD LP. Contributed reagents/materials/analysis tools: DL ST JD LS FL. Wrote the paper: DL ST JD LP.
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