Mouse Survival Motor Neuron Alleles That Mimic SMN2 Splicing and Are Inducible Rescue Embryonic Lethality Early in Development but Not Late

Suzan M. Hammond1,2, Rocky G. Gogliotti1,2, Vamshi Rao1,2, Ariane Beauvais3,4, Rashmi Kothary3,4,5,6, Christine J. DiDonato1,2*

1 Human Molecular Genetics Program, Children’s Memorial Research Center, Chicago, Illinois, United States of America, 2 Department of Pediatrics, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, United States of America, 3 Ottawa Hospital Research Institute, Ottawa, Canada, 4 The University of Ottawa Center for Neuromuscular Disease, Ottawa, Canada, 5 Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Canada, 6 Department of Medicine, University of Ottawa, Ottawa, Canada

Abstract

Spinal muscular atrophy (SMA) is caused by low survival motor neuron (SMN) levels and patients represent a clinical spectrum due primarily to varying copies of the survival motor neuron-2 (SMN2) gene. Patient and animals studies show that disease severity is abrogated as SMN levels increase. Since therapies currently being pursued target the induction of SMN, it will be important to understand the dosage, timing and cellular requirements of SMN for disease etiology and potential therapeutic intervention. This requires new mouse models that can induce SMN temporally and/or spatially. Here we describe the generation of two hypomorphic Smn alleles, Smn[5-7]Neo and Smn[8-8]Neo. These alleles mimic SMN2 exon 7 splicing, titrate Smn levels and are inducible. They were specifically designed so that up to three independent lines of mice could be generated, herein we describe two. In a homozygous state each allele results in embryonic lethality. Analysis of these mutants indicates that greater than 5% of Smn protein is required for normal development. The severe hypomorphic nature of these alleles is caused by inclusion of a loxP-flanked neomycin gene selection cassette in Smn intron 7, which can be removed with Cre recombinase. In vitro and in vivo experiments demonstrate these as inducible Smn alleles. When combined with an inducible Cre mouse, embryonic lethality caused by low Smn levels can be rescued early in gestation but not late. This provides direct genetic evidence that a therapeutic window for SMN inductive therapies may exist. Importantly, these lines fill a void for inducible Smn alleles. They also provide a base from which to generate a large repertoire of SMA models of varying disease severities when combined with other Smn alleles or SMN2-containing mice.

Citation: Hammond SM, Gogliotti RG, Rao V, Beauvais A, Kothary R, et al. (2010) Mouse Survival Motor Neuron Alleles That Mimic SMN2 Splicing and Are Inducible Rescue Embryonic Lethality Early in Development but Not Late. PLoS ONE 5(12): e15887. doi:10.1371/journal.pone.0015887

Editor: Brian D. McCabe, Columbia University, United States of America

Received September 1, 2010; Accepted November 27, 2010; Published December 29, 2010

Copyright: © 2010 Hammond et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The work was funded by the National Institutes of Health [1R01NS060926 and 1R21HD058311] to CJD, the Canadian Institutes of Health Research (CIHR) to RK and the Families of Spinal Muscular Atrophy to CJD. RK is a recipient of a University Health Research Chair from the University of Ottawa. RG is supported by a NIH training grant [T32 AG000260] “Drug Discovery Training in Age-related Disorders.” The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: c-didonato@northwestern.edu

Introduction

The survival motor neuron (SMN) gene is ubiquitously expressed and encodes an essential protein that is required by all cells [1]. Low levels of SMN cause proximal spinal muscular atrophy (SMA), an autosomal recessive disease, and a common genetic cause of infant mortality [2,3]. It is pathologically characterized by selective loss of lower motor neurons within the spinal cord, causing progressive muscle atrophy due to denervation. Proximal muscles within the limbs and trunk are more affected than distal muscle groups, but ultimately all muscles succumb to denervation causing paralysis, respiratory deficiency and ultimately death.

Clinically, SMA is heterogeneous and has been divided into three major groups based upon age at onset and achieved motor milestones [4,5]. Genetically SMA is homogenous in that all forms of the disease are caused by homozygous deletion, rare subtle mutations, or gene conversion of the survival motor neuron-1 (SMN1) gene with concurrent retention of a linked paralog, survival motor neuron-2 (SMN2) [2,6,7]. Both SMN genes reside in a duplicated genomic region at 5q13, are transcribed, translated and 99.9% identical [2,8,9]. The key difference is a single, translationally silent nucleotide transition (C to T) at the +6 position within exon 7 that functionally distinguishes SMN1 from SMN2 and prevents SMN2 from fully compensating for SMN1 loss [2,9,10]. SMN1 contains a “C” nucleotide and produces full-length SMN transcripts (FL-SMN). In contrast, SMN2 contains a “T” nucleotide and primarily produces transcripts that lack exon 7 (SMN7A) and a small amount of FL-Smn. This is due to the simultaneous disruption of an ASF/SF2 exon splice enhancer (ESE) and creation of an exon splice silencer (ESS) in SMN2 [11,12].

The SMN2 copy number in a individual can vary from one to six and it is this variability that is mainly responsible for the clinical spectrum seen in SMA patients [13]. Since every SMA patient has at least one functioning SMN2 gene, it has become a target for therapeutic interventions, and most pre-clinical studies have focused on up-regulating SMN levels by some means.
An important point of all SMN-dependent therapies is an understanding of when, where and how much SMN induction is required, and how this might change for the various clinical forms of SMA. The dosage, timing and cellular requirements of SMN in different tissues should not be overlooked as there is mounting evidence in humans and mice that suggest non-motor neuron targets such as heart, autonomic and vascular systems may require consideration [25,26,27,28]. Although some data is already available and demonstrates a therapeutic window of opportunity to affect a benefit for severe SMA mice [15,17,29], a new panel of mice is required in which SMN can be induced temporally and/or spatially to refine and extend current results.

In this study, we report the generation and characterization of two Smn progenitor alleles, SmnC-T-Neo and Smn2B-Neo. They were designed to stimulate Smn exon 7 alternative splicing, which normally does not occur in the mouse [30,31]. SmnC-T-Neo and Smn2B-Neo are severe hypomorphs that cause embryonic lethality when in a homozygous state due to the presence of a loxP-flanked neomycin (neo) gene resistance cassette that hinders Smn expression. However, in the presence of Cre recombinase, the embryonic lethality can be rescued by neo excision, while still maintaining Smn exon 7 alternative splicing via our introduced mutations. In vitro and in vivo experiments demonstrate the utility of these mice to be used as inducible Smn alleles when combined with Cre transgenic lines. Using a tamoxifen-inducible Cre line we show that embryonic lethality can be rescued early in gestation but not late. As a final point, the embryonic lethality can be rescued by neo excision, while still maintaining Smn exon 7 alternative splicing via our introduced mutations. In vitro and in vivo experiments demonstrate the utility of these mice to be used as inducible Smn alleles when combined with Cre transgenic lines. Using a tamoxifen-inducible Cre line we show that embryonic lethality can be rescued early in gestation but not late.

**Results**

**Generation and germline transmission of Smn<sup>C-T</sup>-Neo and Smn<sup>2B</sup>-Neo alleles**

Based on our previous studies we designed two replacement vectors, p(SmnC-T-Neo) and p(Smn2B-Neo) and introduced two different mutations into the endogenous Smn locus by homologous recombination. The first mimics human SMN2 and is a C-T transition at position 6 of exon 7, referred to hereafter as the C-T mutation. The second mutation alters the central portion of the ESE where hTra2-Beta1 binds Smn exon 7 (GGA to TTT), and we refer to this mutation as the 2B mutation [32] (Figure 1A). It is known that this binding site is important for SMN exon 7 processing [33]. For both replacement vectors the positive selection cassette, pgk-neo, was inserted into the unique BamHI site ~180 bp distal to exon 7 in the antisense orientation to Smn transcription. This was specifically done as an additional way to potentially hinder Smn processing. We also flanked ("Boxed") the pgk-neo cassette with loxP sites so that it could be excised with Cre recombinase in future experiments to leave the endogenous locus with a minimal amount of alteration.

Homologous recombiant embryonic stem cell clones were identified by Southern blot hybridization (Figure 1B) and two independent clones for each construct used to generate chimeras. Germline transmission was confirmed through direct sequencing of Smn exon 7 PCR products (Figure 1C). We refer to these progenitor lines as Smn<sup>C-T</sup>-Neo (official name Smn<sup>1tm2Cdid</sup>) and Smn<sup>2B</sup>-Neo (official name Smn<sup>2Bneo</sup>) as they retain the floxed pgk-neo selection cassette within Smn intron 7. All subsequent experiments extend current results.

![Figure 1. Generation of mutant Smn alleles.](https://example.com/figure1.png)

**Figure 1. Generation of mutant Smn alleles.** (A) Gene targeting strategy to introduce the C-T and 2B mutation into Smn exon 7 using the gene targeting vectors p5mC-T-Neo and p5m2B-Neo. (B) Southern blot analysis of BamHI and PstI digested DNA from neo resistant E15 cell clones identified homologous recombinants. Two clones from each were used to perform blastocyst injections. (C) Germline transmission of Smn<sup>C-T</sup>-Neo and Smn<sup>2B</sup>-Neo alleles were determined by direct sequencing of Smn exon 7 PCR products from heterozygous mice. The C-T mutation corresponds to the nucleotide transition within exon 7 of the SMN2 gene. The 2B mutation corresponds to a mutation within the splice enhancer region 2B, changing GGA to TTT [32].

doi:10.1371 journal.pone.0015887.g001
Snm\(^{C\text{-T-Neo}}\) and Snm\(^{2B\text{-Neo}}\) alleles express very small amounts of Smn

To evaluate the effects of C-T-Neo and 2B-Neo mutations on Snm exon 7 processing, all major organs including spinal cord and skeletal muscle from postnatal tissues of heterozygous mice were analyzed by reverse transcription-polymerase chain reaction (RT-PCR). Snm\(^{C\text{-T-Neo/WT}}\) mice produced both FL-Smn and ATSmn transcripts as did Snm\(^{2B\text{-Neo/WT}}\) mice (data not shown). We performed a series of intercrosses for each allele during the course of this study. Of 229 and 281 pups genotyped at weaning from Snm\(^{C\text{-T-Neo/WT}}\) and Snm\(^{2B\text{-Neo/WT}}\) intercrosses, respectively, none were found to be homozygous (Table 1). To identify when during development Snm\(^{C\text{-T-Neo/C-T-Neo}}\) and Snm\(^{2B\text{-Neo/2B-Neo}}\) embryos were dying, we performed a series of timed matings. For Snm\(^{C\text{-T-Neo/WT}}\) and Snm\(^{2B\text{-Neo/WT}}\) intercrosses at 9.5 days post-coitum (E9.5), a total of 58 embryos were analyzed. While near mendelian ratios of wild type (Smn\(^{WT/WT}\)) homozygous (Smn\(^{C\text{-T-Neo/C-T-Neo}}\)) and homozygous mutants (Smn\(^{C\text{-T-Neo/C-T-Neo}}\)) were identified by genotyping, developmental delays of homozygous mutant Smn\(^{C\text{-T-Neo/C-T-Neo}}\) embryos (11/58; 19%) were visible (Table 1 and Figure 2, panels a–h). Although at E12.5 we could still detect Smn\(^{C\text{-T-Neo/C-T-Neo}}\) embryos (5/56; 9%), it was clear that all were developmentally delayed and at different levels of resorption (Table 1 and Figure 2 panels a’–p’). By E15.5 only wild type and heterozygous Smn\(^{C\text{-T-Neo/WT}}\) embryos were present (Table 1).

Our results for embryo analysis from Snm\(^{2B\text{-Neo/WT}}\) intercrosses were more dramatic. We analyzed 84 embryos at E9.5 and while we could detect Smn\(^{2B\text{-Neo/2B-Neo}}\) homozygotes (14/84; 17%), these embryos were more developmentally delayed than the Smn\(^{C\text{-T-Neo/C-T-Neo}}\) embryos and were starting to be reabsorbed (Table 1 and Figure 2 panels a’–h’). At E12.5 all Smn\(^{2B\text{-Neo/2B-Neo}}\) homzygotes that we could detect were dead and at different levels of resorption (Table 1 and Figure 2, panels i’–p’). By E15.5 only wild type and heterozygous Smn\(^{2B\text{-Neo/WT}}\) embryos were present (Table 1).

The embryonic lethality of Smn\(^{C\text{-T-Neo/C-T-Neo}}\) and Smn\(^{2B\text{-Neo/2B-Neo}}\) embryos occurs later than Smn\(^{AT\text{Smn}}\) embryos, which die at E7.5 [34,35]. This suggested that a small amount of FL-Smn was being produced. To determine whether this was the case, we analyzed RNA from E9.5 wild type, heterozygous and homozygous Smn\(^{C\text{-T-Neo/WT}}\) and Smn\(^{2B\text{-Neo/WT}}\) embryos by RT-PCR. Both FL-Smn and ATSmn were produced from heterozygous and homozygous Smn\(^{C\text{-T-Neo/WT}}\) and Smn\(^{2B\text{-Neo/WT}}\) embryos in contrast to wild type Smn embryos (Smn\(^{WT}\)) (Figure 3A). Furthermore, the Smn\(^{C\text{-T-Neo}}\) allele consistently produced more transcripts that contained Smn exon 7 than the Smn\(^{2B\text{-Neo}}\) allele. Direct sequencing of the FL-Smn amplicon from Smn\(^{C\text{-T-Neo/C-T-Neo}}\) embryos identified only the mutant “T” nucleotide at position +6 of Smn exon 7, as would be expected from homozygous mutant embryos (Figure 3B). Likewise, FL-Smn amplicons from Smn\(^{2B\text{-Neo/2B-Neo}}\) embryos only expressed the 2B (TTT) mutation (data not shown).

To quantify the amount of FL-Smn and ATSmn transcripts that were produced in each of the genotypes from our intercross experiments, we designed two novel taqman assays for use in quantitative reverse transcription PCR (qRT-PCR). The first assay specifically detected Smn exon 7 in the presence of either the wild type, C-T or 2B mutation with similar efficiency. The second assay detected ATSmn transcripts. The values of FL-Smn from our Smn\(^{C\text{-T-Neo}}\) and Smn\(^{2B\text{-Neo}}\) genotypes were compared to the expression of Smn\(^{WT}\) E9.5 embryos derived from the same intercross to reduce variability. Overall, the amount of FL-Smn varied with our different genotypes (Figure 3C and Table 2). Smn\(^{C\text{-T-Neo/C-T-Neo}}\) embryos produced 20±5% FL-Smn, whereas Smn\(^{2B\text{-Neo/2B-Neo}}\) embryos produced 15±3% and these values were not statistically significant from each other (p=0.43). This indicated that each Smn\(^{C\text{-T-Neo}}\) and the Smn\(^{2B\text{-Neo}}\) allele produced ~10% and ~7.5% FL-Smn transcripts, respectively. These results are consistent with Smn\(^{C\text{-T-Neo/WT}}\) 62±12% and Smn\(^{2B\text{-Neo/WT}}\) 59±7% embryos, if you consider that ~50% of FL-Smn transcripts are derived from the Smn\(^{WT}\) allele (Figure 3C and Table 2).

We also quantified the amount of ATSmn from our Smn\(^{C\text{-T-Neo}}\) and Smn\(^{2B\text{-Neo}}\) genotypes by comparing them to ATSmn transcripts derived from spinal cord samples of Smn\(^{AT\text{Smn}}\) mice [34]. Smn\(^{2B\text{-Neo/2B-Neo}}\) embryos expressed the greatest amount of ATSmn transcripts, whereas the Smn\(^{C\text{-T-Neo/WT}}\) embryos expressed the least (Figure 3A and 3C). Using this data in conjunction with our FL-Smn data we were able to generate a FL-Smn: ATSmn ratio for each genotype using the ΔΔCt values. The ratio for Smn\(^{AT\text{Smn/WT}}\) mice was approximately equal to 1.0 (1.02±0.018) and served as a control. The wild type allele (WT) only produces transcripts that contain Smn exon 7, whereas the Smn\(^{AT\text{Smn}}\) allele only produces transcripts that lack exon 7 due to the absence of the exon in the genome of this allele [34]. In comparison, Smn\(^{C\text{-T-Neo/WT}}\) mice produced about 12-fold more (11.98±0.929) FL-Smn than ATSmn transcripts. However, in homozygous embryos (Smn\(^{C\text{-T-Neo/C-T-Neo}}\)) the ratio was almost equal to 1.0 (0.989±0.068) and this is consistent with

---

**Table 1. Genotype of Snm\(^{C\text{-T-Neo}}\) and Snm\(^{2B\text{-Neo}}\) intercross progeny.**

| Age    | # of Litters | Total Pups | +/- (%) | +/- (%) | -/- (%) | unknown identity (%) | \( \chi \) |
|--------|--------------|------------|---------|---------|---------|----------------------|--------|
| **Snm\(^{C\text{-T-Neo}}\) intercross progeny** | | | | | | | |
| 1 month | 39 | 229 | 80 (35) | 144 (63) | 0 (0) | 5 (2) | 83.6 (p<0.001) |
| E15.5 | 5 | 33 | 15 (45) | 18 (54.5) | 0 (0) | 0 (0) | 13.9 (p<0.001) |
| E12.5 | 8 | 56 | 14 (25) | 28 (50) | 5 (9) | 9 (16) | 5.17 (p>0.05) |
| E9.5 | 6 | 58 | 16 (27.5) | 22 (38) | 11 (19) | 9 (15.5) | 1.5 (p<0.05) |
| **Snm\(^{2B\text{-Neo}}\) intercross progeny** | | | | | | | |
| 1 month | 44 | 281 | 109 (39) | 170 (60.5) | 0 (0) | 2 (0.7) | 98.5 (p<0.001) |
| E15.5 | 6 | 44 | 22 (50) | 22 (50) | 0 (0) | 0 (0) | 22 (p<0.001) |
| E12.5 | 7 | 63 | 5 (8) | 36 (57) | 9 (14) | 13 (21) | 116 (p<0.01) |
| E9.5 | 9 | 84 | 21 (25) | 38 (45) | 14 (17) | 11 (13) | 1.46 (p>0.05) |

(+) denotes wild type, (−) denotes either C-T-Neo or 2B-Neo.

doi:10.1371/journal.pone.0015887.t001
Figure 2. Whole mount analysis of embryos. SmnC-T-Neo or Smn2B-Neo heterozygotes were intercrossed and embryos obtained at either E9.5 or E12.5 for whole-mount analysis and genotyping. (a–h) E9.5 SmnC-T-Neo littermates. Homozygotes (C-T-N/C-T-N) are small but alive and larger than the Smn1-C-T-Neo/2B-Neo (2B-N/2B-N) homozygotes. (i–p) E12.5 SmnC-T-Neo embryos. Homozygotes are extremely small compared to controls and many are being reabsorbed as shown in panel (p). (a’–h’) E9.5 Smn2B-Neo embryos. Heterozygotes (2B-N/WT) are identical to wild type (WT/WT) littermates. Homozygotes (2B-N/2B-N) are developmentally retarded though still alive with signs of lethality clearly present before this period in some embryos that did not allow for genotyping (Table 1). (i’–p’) E12.5 Smn2B-Neo embryos. All homozygous mutant embryos are undergoing resorption. Insets in o’ and p’ are magnified images of embryos in panel. Scale for all E9.5 embryos is 100 μM and for E12.5 200 μM.

doi:10.1371/journal.pone.0015887.g002

the visual inspection of end-point RT-PCR (Figure 3A). Interestingly, we found the FL-Smn:Δ7Smn ratio to be 10-fold less in Smn2B-Neo/2B-Neo embryos (0.178±0.022) as compared to SmnC-T-Neo/C-T-Neo embryos even though the amount of FL-Smn produced from either the SmnC-T-Neo or Smn2B-Neo allele was not significantly different (p = 0.43). The change in ratio is due to the high levels of Δ7Smn transcripts that SmnC-T-Neo/WT embryos produced and is consistent with the splicing pattern and transcript ratios of SMN2 where the majority of SMN2 transcripts lack exon 7.

To correlate FL-Smn transcripts to Smn protein we performed western blot analysis of single E9.5 embryos for each possible genotype. We had difficulty detecting Smn in homozygous mutant embryos, and the level varied between mutants of the same genotype (Figure 3D). In general, the level of Smn correlated with the severity of the mutant embryo when we retrospectively compared Smn levels across the array of mutants to our whole mount mutant embryo images (Figure 3D and Figure 2). The level of Smn protein in SmnC-T-Neo/C-T-Neo embryos ranged from 2–5% and in Smn2B-Nos/2B-Nos embryos it was about 1–3%. This was an unexpected finding based on our FL-Smn expression data from SmnC-T-Neo/C-T-Neo and Smn2B-Nos/2B-Nos embryos and was most likely caused by the compromised physiological state of the SMA embryos, a lack of stability of higher order Smn complexes from low Smn levels and transcriptional and/or translational hindrance from the floxed pgk-neo cassette. Since the later was the only one that we could directly test, we removed the floxed pgk-neo cassette from the germline using homozygous EIIa-Cre transgenic mice that ubiquitously express Cre recombinase very early in embryogenesis [36]. This increased Smn protein levels from <5% for homozygous SmnC-T-Neo and Smn2B-Nos embryos to >30% for a single SmnC-T-Neo allele and ~16% for a single Smn2B allele (data not shown and will be reported elsewhere).

Collectively, these results confirm that our SmnC-T-Neo and Smn2B-Nos alleles are severe hypomorphs. They express very low amounts of Smn due to the nature of our introduced mutations as well as the presence of a floxed pgk-neo selection cassette that hinders Smn expression. These alleles are “repairable” and have the potential to be used as inducible Smn alleles that mimic SMN2 splicing. To determine this directly, the following in vitro and in vivo experiments were designed to address the ability of SmnC-T-Neo and Smn2B-Nos alleles to be utilized as inducible Smn alleles by excision of the floxed pgk-neo cassette.

Induction of Smn expression in vitro

We determined the ability of our progenitor alleles to induce Smn levels through excision of the floxed pgk-neo cassette in vitro using primary murine embryonic fibroblasts (MEFs). For these experiments, we used our SmnC-T-Neo allele in combination with a tamoxifen (TM) inducible Cre allele, CreEsr1[37]. We established and cultured MEF lines from two SmnC-T-Neo/WT;CreEsr1 embryos and compared untreated MEF cultures to those treated with TM. Excision of floxed pgk-neo by CreEsr1 was monitored at the DNA level by three primer PCR analysis (Figure 4A). The reaction amplified all three possible Smn alleles: WT (470 bp), C-T-Neo
Figure 3. Smn transcript analysis analysis of E9.5 embryos. (A) RT-PCR of E9.5 embryos comparing wild type, heterozygotes, and homozygotes. Both FL-Smn and Δ7Smn transcripts are amplified from cDNA of mice that are heterozygous and homozygous for the mutant alleles. (B) Direct sequencing of FL-Smn and Δ7Smn RT-PCR products derived from SmnC-T-Neo/C-T-Neo mutants. The “T” denoted with an arrow above it, represents the C-T mutation. Dotted line within exon6-8 sequence represents the junction between exon 6 and exon 8. (C) qRT-PCR results of E9.5 embryo for FL-Smn and Δ7Smn transcripts. FL-Smn transcripts for C-T-Neo and 2B-Neo were compared to wild type and heterozygous embryos within their own intercrosses and litters to control for variability. Spinal cord (S.C.) cDNA from a 6-month Δ7/WT mouse was used to compare Δ7Smn transcripts from C-T-Neo and 2B-Neo heterozygous and homozygous embryos. Wild type mice do not express Δ7Smn and are not shown on the graph. Each data point on the graphs represent individual embryos and depiction of variability between embryos. (D) Immunoblot analysis of Smn expression from individual E9.5 embryos derived from SmnC-T-Neo or Smn2B-Neo intercrosses. 15 times less protein was used for the controls to avoid overloading SMN while simultaneously detecting it in the mutants. Note the variation in Smn levels from individual mutant embryos. Lower Smn levels correlated with more severe phenotypes (see Figure 2). To achieve this sensitivity, Smn detection was performed on a Li-COR Odyssey infrared imaging system. Abbreviations: (WT) SmnWT/WT, (C-T-N/WT) SmnC-T-Neo/WT, (2B-N/WT) Smn2B-Neo/WT, (C-T-N/C-T-N) SmnC-T-Neo/C-T-Neo, (2B-N/2B-N) Smn2B-Neo/2B-Neo, (Δ7/WT) SmnΔ7/WT.

doi:10.1371/journal.pone.0015887.g003
(500 bp) and C-T (577 bp) (Figure 5B). MEFs treated with TM demonstrated excision of the floxed pgk-neo cassette from the SmnC-T-Neo allele (Figure 4B, lanes 4 and 5). A low level of excision was also noted in cells without TM treatment; we attributed this to the previously reported <0.1% spontaneous Cre activity that was reported for this line [37] (Figure 4B, lanes 2 and 3). We then correlated pgk-neo excision with an increase in FL-Smn expression by directly sequencing RT-PCR products from treated and untreated MEFs. A clearly discernable “T” nucleotide was present in those cells treated with TM and absent in the untreated cultures indicating an increase of Smn expression specifically from our mutant allele (Figure 4C). This was substantiated and quantified by qRT-PCR analysis which showed an 83% increase in FL-Smn transcripts and a 30% reduction in Δ7Smn transcripts between treated and untreated cultures (Figure 4D). Therefore, addition of tamoxifen to cultures of SmnC-T-Neo/WT, CreERT2 MEFs increased FL-Smn expression through excision of the floxed pgk-neo cassette in Smn intron 7 and demonstrates the inducible nature of these alleles in vitro.

The ability of our Smn alleles to be Cre responsive in post natal somatic tissues was determined by single intraperitoneal (i.p.) injections [9 mg/40 g body weight] of TM or vehicle (corn oil) to 2–4 month old SmnC-T-Neo/WT, CreERT2 mice. They were euthanized five days post-injection and DNA from kidney, spinal cord, skeletal muscle, forebrain and cerebellum were used as template in PCR to determine floxed pgk-neo excision. Vehicle injected SmnC-T-Neo/WT, CreERT2 mice had a low level of DNA excision consistent with background levels of CreERT2 (Figure 5A, lane 2). SmnC-T-Neo/WT, CreERT2 mice without CreERT2 showed no excision of the floxed pgk-neo cassette (Figure 5A, lane 3). In contrast, excision occurred in all tissues examined from SmnC-T-Neo/WT, CreERT2 mice after a single i.p. injection (Figure 5A, lane 4), hence somatically changing the SmnC-T-Neo allele to a SmnC-T allele.

Induction of Smn in SmnC-T-Neo/C-T-Neo, CreERT2 embryos early, but not late, rescues embryonic lethality

The early lethality of SmnC-T-Neo/C-T-Neo embryos was determined to be caused by low Smn levels, thus we next sought to determine when during development, if at all, we could increase Smn levels and rescue the embryonic lethality phenotype. To this end we crossed SmnC-T-Neo/WT females to SmnC-T-Neo/WT, CreERT2 males in order to produce SmnC-T-Neo/C-T-Neo, CreERT2 embryos. Without TM

### Table 2. qRT-PCR analysis of E9.5 Smn embryos.

| FG5 embryos | FL-Smn expression | Δ7 Smn expression |
|-------------|-------------------|------------------|
| WT/WT       | 1.00 ± 0.02       | 0.46 ± 0.14      |
| C-T-Neo/WT  | 0.62 ± 0.12       | 0.33 ± 0.06      |
| 2B-Neo/WT   | 0.59 ± 0.07       | 1.72 ± 0.23      |
| C-T-Neo/CT-Neo | 0.20 ± 0.05 | 1.56 ± 0.22      |
| 2B-Neo/2B-Neo | 0.15 ± 0.03   | 1.24 ± 0.32      |
| Δ7/WT (6 month S.C.) | 1.00 ± 0.10 |                |

S.C., spinal cord.

doi:10.1371/journal.pone.0015887.t002

**Figure 4. Smn expression is efficiently induced from the SmnC-T-Neo allele in vitro.** Two independent primary MEF cell lines were derived from double transgenic embryos (SmnC-T-Neo/WT, CreERT2). (A) Schematic of the SmnC-T-Neo allele from exon 6 to exon 8. Arrows represent forward and reverse primers used in the 3-plex PCR reaction to identify SmnC-T-Neo (640 & 637), SmnC-T-Ne (638 & 637), and SmnC-T (640 & 637) alleles. Primers 640 and 637 do not amplify a product in the presence of pgk-neo as the amplicon exceeds the time of elongation. (B) 3-plex PCR amplification of DNA from MEF lines 1 and 2 treated for 1 hr with 1 mM tamoxifen (+TM). MEF lines 1 and 2 left untreated (-TM) showed a slight amount of background excision (lanes 2 & 3); however, in the presence of tamoxifen (+TM), they readily amplify the SmnC-T allele (lanes 4&5). Controls in lanes 6, 7, and 8 were E10.5 SmnC-T-Neo/+ backgrounds. In lanes 6, 7, and 8 were E10.5 embryos harvested to show the indicated genotypes from crosses using germline transmitting SmnC-T-Neo and SmnC-7 alleles. (C) DNA from untreated (-TM) and induced (+TM) MEF cells were amplified by RT-PCR and FL-Smn transcripts directly sequenced. Induced MEFs (+TM) produced enough FL-Smn transcripts from the mutant C-T allele that could be detected by direct sequencing. The arrow points out the C-T mutation in +TM treated cultures. (D) qRT-PCR of FL-Smn and Δ7Smn from uninduced (-TM) and induced (+TM) cultures. Abbreviations: (TM) tamoxifen (C-T/N/WT, CreERT2) SmnC-T-Neo/WT, SmnC-T-Ne/WT (C-T/N/WT) SmnC-T-Neo/C-T (C-T/N/C-T) SmnC-T-Neo/C-T.

doi:10.1371/journal.pone.0015887.g004

PLoS ONE | www.plosone.org 6 December 2010 | Volume 5 | Issue 12 | e15887
treatment these embryos should undergo complete resorption between E12.5 and E15.5 (Figure 2 and Table 1). Pregnant females were injected with TM [3 mg/40 g body weight] at either E7.5 or E13.5. Only two litters (9 pups total) survived birthing from 7 dams injected at E7.5 and visibly pregnant at late gestation. Of these 9 pups, 1 was a SmnC-T-Neo/C-T-Neo;CreEsr1 pup and of similar size and appearance to its littermates. However, in both litters the dams failed to lactate and fostering was unsuccessful. Birthing and lactation problems are a known common side effect of TM administration, especially when given early during pregnancy [37]. To avert this problem we harvested embryos at E18.5, a late point in gestation, and well after a stage in development (E15.5) in which the embryos should have undergone complete resorption. 

Figure 5. Smn induction in adults and embryos from a single injection of tamoxifen. (A) DNA analysis of adult mice i.p. injected with vehicle (corn oil) or TM (9 mg/40 g body weight) using the same 3-plex PCR reaction as shown in Figures 4A and B. Wild type mice (WT;Cre-) only amplified the wild type allele (lane 1). Doubly transgenic mice (SmnC-T-Neo/WT;CreEsr1) in the absence of TM (-TM) displayed a low basal level of pgk-neo excision as has been previously reported for this Cre line [37]. In the absence of the CreEsr1 transgene, SmnC-T-Neo/WT mice injected with TM could not excise pgk-neo (lane 3), in all tissues analyzed, pgk-neo excision was only possible and efficient in the presence of CreEsr1 and TM (lane 4). (B) PCR analysis of E18.5 embryos that received a single i.p. dose of TM (3 mg/40 g body weight) to the pregnant dam at E7.5 or E13.5 DNA was genotyped as above to differentiate SmnWT, SmnC-T-Neo and SmnC-T alleles. Arrows identify the appropriate amplicons. (C) Photomicrograph of E18.5 embryos induced with TM at E7.5 or E13.5. Lines in photograph show where images were tiled together in Photoshop. (D) Western blot and semi-quantitative densitometry of protein extracted from brain tissue of induced and control E18.5 embryos. A small amount of protein was able to be extracted from severely deformed SmnC-T-Neo/C-T-Neo embryos identified as "escapers" for comparison to induced SmnC-T-Neo/C-T-Neo;CreEsr1 rescued embryos. Semi-quantitative densitometry was performed on a separate blot using the same samples shown and normalized to β-tubulin, without the uninduced mutant. Protein levels from induced homozygous embryos, SmnC-T-Neo/C-T-Neo;CreEsr1, (0.7±0.10) was greater than SmnWT (0.5±0.2). Abbreviations: (WT) Smn wild type allele, (Cre+ and Cre-) presence or absence of CreEsr1, (C-T-N/WT) SmnC-T-Neo/WT, (C-T-N/C-T-N) SmnC-T-Neo/C-T-Neo, (C-T) SmnC-T allele, (C-T-Neo) SmnC-T-Neo allele, (TM) tamoxifen. doi:10.1371/journal.pone.0015887.g005
which no viable homozygous Smn<sup>C-T-Neo/C-T-Neo</sup> embryos had previously been identified (Table 1). DNA prepared from yolk sacs or tails was used for PCR-based genotyping with the same 3-plex PCR assay used in Figures 4 and 5A. This allowed us to identify and determine the efficiency of the floxed pgk-neo cassette excision. The presence or absence of the Cre<sup>Esr1</sup> allele and Cre<sup>Esr1</sup> transgene, the injection of TM induced excision of floxed pgk-neo, thus systematically changing the Smn<sup>C-T-Neo</sup> allele to a Smn<sup>C-T</sup> allele (Figure 5B, lanes 5-9). Smn induction of 4 litters at E7.5 resulted in a total of 32 embryos at E18.5 and five were found to be Smn<sup>C-T-Neo/C-T-Neo</sup> or Smn<sup>C-T</sup> at E18.5 (Table 3). All five of these embryos were viable, of similar size and indistinguishable from control embryos (Figure 5C). In contrast, TM injection of 6 litters at E13.5 resulted in no viable Smn<sup>C-T-Neo/C-T-Neo</sup> or Smn<sup>C-T</sup> embryos at E18.5 (0/42) (Table 3). However, in each of these cases we did identify a Smn<sup>C-T-Neo/C-T-Neo</sup> embryo that was negative for the Cre<sup>Esr1</sup> transgene although both were severely deformed, nonviable and undergoing resorption (Figure 5C). Chi-square statistics were used to compare the genetic ratios of induced embryos to expected normal mendelian ratios with the assumption that Smn<sup>C-T-Neo/C-T-Neo</sup> embryos would not be present at E18.5. Genotype ratios from embryos treated at E13.5 were not statistically significant from expected ratios, p = 0.84. However, the genotype ratios of embryos treated at E7.5 were significant, p = 0.05 (Table 3). Thus, increasing Smn levels rescues the embryonic lethality phenotype of Smn<sup>C-T-Neo/C-T-Neo</sup> and Cre<sup>Esr1</sup> embryos when induced early, but not late in development.

To quantify the level of Smn in these Smn<sup>C-T-Neo/C-T-Neo</sup> or Cre<sup>Esr1</sup> embryos, we performed western blot analysis and compared them to a control, age-matched embryos that over Smn levels between 100% and 50%. In addition, we were able to extract a small amount of protein from one of the non-viable Smn<sup>C-T-Neo/C-T-Neo</sup> or Cre<sup>Esr1</sup> embryos for comparison to our induced embryos (Figure 5D). However, with Cre mediated excision of the floxed pgk-neo cassette, Smn levels in this embryo were below the limit of detection even though the loading control, β-tubulin was visualized (Figure 5D). Densitometry was performed on a separate western blot using the same samples for the c-myc experiment. c-myc was expressed in our embryos (Figure 5D) [1]. Furthermore, this was the greater than the level of Smn expressed in our embryos and Smn<sup>2B</sup> progenitor lines of mice that have a normal lifespan and are without phenotype.

**Discussion**

We specifically designed the targeting vectors used in this study to have a triple function so that a series of Smn alleles could be generated. The first was the introduction of mutations within known Smn exons 7 ESEs to mimic SMN2 exon 7 splicing and attenuate Smn expression. The second was the placement of the positive selection cassette within intron 7 with the aim of further diminishing the amount of Smn generated from these targeted alleles. It has previously been shown that selection cassettes located within introns or regulatory regions, such as pgk-neo that we used here, can hinder expression of targeted genes via transcriptional and/or translation interference either by design [53]; additional examples cited in [39] or inadvertently [40,41,42], which sometimes proves to be serendipitous [42]. The level of interference can be varied depending upon the orientation of the selection cassette if it has cryptic splice donor and acceptor sites, like the neo gene, [39] or they can be engineered. Here we used pgk-neo in the inverted orientation to Smn transcription as the cryptic splice sites were stronger. The third and final function of our design was to flank pgk-neo with loxP sites so that it could be removed with Cre recombinase. This allows the specific analysis of our introduced mutations within the context of a minimally altered genomic locus. Since the neo cassette hindered Smn expression as we designed, our alleles can be used to induce Smn expression while still mimicking SMN2 exon 7 alternative splicing. The conceptual properties of this targeting design are applicable to almost any gene targeting strategy and warrant consideration for those embarking on new projects.

Our allelic targeting strategy provides the opportunity to generate three mouse lines from each single allele. The first is the original progenitor alleles, Smn<sup>C-T-Neo</sup> and Smn<sup>2B</sup>. The second is these alleles in combination with a tissue specific or inducible Cre transgene for future time and cell-specific induction experiments. Finally, mouse lines possessing the point mutations themselves, Smn<sup>C-T</sup> and Smn<sup>2B</sup>, can be produced. Here we have focused on characterizing the first two lines of mice.

Mice heterozygous for the Smn<sup>C-T-Neo</sup> and Smn<sup>2B</sup> alleles were normal. When homozygous, these alleles caused embryonic lethality, even though full-length transcripts and small amounts of Smn protein could be detected. This result illustrates once again that a minimum amount of Smn is required by all cell types [1,34,55,43,44], and this appears to be about 5% during development in mice. Although we found no significant difference in mendelian ratios of homozygous mutants for either allele at E9.5, morphologically it was clear that the embryos were dead or extremely growth retarded and their ability to develop corresponded to their Smn levels. Embryonic lethality during this period of development is commonly caused by potential failures in vasculogenesis or hematopoiesis and/or cardiovascular failure [45]. Considering these possibilities, we were able to visualize swollen pericardial sacs in several of our Smn<sup>C-T-Neo/C-T-Neo</sup> homozygous mutants at E11.5 (data not shown) or E12.5 that were not grossly deformed.

**Table 3. Genotypes of Smn embryos at E18.5 exposed to tamoxifen at E7.5 or E13.5.**

| # of litters | Total pups | WT (%) | WT/Cre (%) | C-T-N/WT (%) | C-T-N/WT or Cre (%) | C-T-N/C-T-N (%) | C-T-N/C-T-N (%) | Unknown (%) |
|-------------|-----------|--------|-----------|--------------|---------------------|-----------------|-----------------|------------|
| E7.5 injection, E18.5 harvest | 4          | 32     | 3 (9)     | 5 (16)       | 3 (9)               | 11 (34)         | 1 (3)           | 5 (16)     | 4 (13)     |
| E13.5 injection, E18.5 harvest | 6          | 42     | 6 (14)    | 6 (14)       | 10 (24)             | 10 (24)         | 1 (2)           | 0 (0)      | 9 (21)     |

(χ², E7.5 injection p = 0.051; E13.5 injection p = 0.84). 
doi:10.1371/journal.pone.0015887.t003
Functionally and molecularly the homozygous Smn<sup>C-T-Neo</sup> and Smn<sup>2B-Neo/2B-Neo</sup> hypomorphs are less severe than Smn<sup>A7/47</sup> homozygotes as they express small amounts of fully functional Smn protein and live slightly longer (E12 and E9 vs E7) [34,35]. The Smn<sup>C-T-Neo/2B-Neo</sup> homozygotes are generally less severe than the Smn<sup>2B-Neo/2B-Neo</sup> homozygotes due to the nature of the introduced mutations within exon 7 since the C-T mutation produces more FL-Smn transcripts than the 2B mutation. During the course of our molecular analyses there were two unexpected observations. First, we found that A7Smn transcripts from Smn<sup>2B-Neo/2B-Neo</sup> embryos were extremely high (12.44±3.44) vs. heterozygous Smn<sup>2B-Neo</sup> embryos (1.72±0.23). This may seem odd at first glance, but SMN together with other proteins functions in the assembly of small nuclear ribonucleoproteins (snRNPs), which are critical for pre-mRNA splicing [46,47]. It has previously been demonstrated that low levels of SMN cause changes in snRNP abundance and splicing alterations [48,49,50], so it is possible that SMN regulates its own splicing of SMN exon 7. In fact, Jodelka et al. (2010) [51] has recently identified a feedback loop in which low SMN levels exacerbate SMN exon 7 skipping. This result helps explain the high degree of A7Smn transcripts from our Smn<sup>2B-Neo/2B-Neo</sup> embryos since they produce only 1-3% Smn protein. The second observation was the low amount of Smn protein (<5%) as compared to FL-Smn transcripts (10-20%) in our homozygous embryos. We predict this is caused by a combination of the degenerative state of the embryos, lack of higher order Smn complexes that can stabilize the protein, and the presence of the floxed pgk-neo cassette (as we designed), which creates hybrid Smn/neu transcripts and exerts transcriptional and translational repression. We show that removal of the pgk-neo cassette is able to relieve this repression.

As progress in developing SMN-dependent therapies moves forward, our understanding of when and where SMN is required becomes more important. This can be addressed using animal models since they can be genetically manipulated. SMN has already been modeled in a number of different organisms and each has its strengths [52,53], but the most widely used to date have been SMA mouse models. SMA has already been modeled in a number of different organisms and each has its strengths. SMA mouse models. SMA has already been modeled in different organisms and each has its strengths [52,53], but the most widely used to date have been SMA mouse models. The mouse models fall into two categories: 1) transgenics bred onto a number of different organisms and each has its strengths [52,53], but the most widely used to date have been SMA mouse models.

In a final experiment, we addressed whether the embryonic lethality of Smn<sup>C-T-Neo/2B-Neo</sup> embryos could be rescued. We found that if induction occurred early, around gastrulation (E7.5), but not late (E13.5), and the mutant embryos that were induced at E7.5 expressed ~70% Smn protein. This level of Smn protein is well above that of Smn<sup>+/−</sup> mice that have a normal lifespan [1] or heterozygous SMA model mice [(SMN2)<sub>Abb לימב</sub>+/−; Smn<sup>+/−</sup> Jax strain 5024], which have no motor neuron loss and a normal lifespan [54]. However, this indicates that the embryos are viable and can be induced to express Smn protein at different stages of development.

This can be achieved through genetic crosses of tissue-specific Cre lines or as shown here, through injections if the Cre lines are tamoxifen inducible. In addition, if the Smn<sup>C-T-Neo</sup> and/or Smn<sup>2B-Neo</sup> is used as the Smn mutant background in combination with SMN2 transgenic mice to achieve postnatal survival, new SMA models can potentially be generated with varying degrees of severity. Thus, the timing requirements of Smn inducitive therapies could be addressed postnatally in severe, intermediate and mild forms of SMA. We are currently working to develop these models and determine this.

In conclusion, the gene targeting strategy that we utilized is applicable to almost all gene targeting projects. The strategy provides the potential to generate multiple lines of mice, including an inducible allele, from a single targeting event for the cost of extra effort put into the planning stage of vector design. As proof, we generated an allelic series of Smn mice. They produce small amounts of Smn, mimic SMN2 splicing and are inducible. While these are the first inducible Smn alleles to be described in the literature, other groups are also working to generate inducible Smn mice using different strategies such as Tet-on/off systems or other Cre-inducible alleles. As proven with the various transgenic models of SMA, all will play important and complimentary roles as we move forward to understand Smn function in health and disease, and progress towards developing a therapy for SMA.

### Materials and Methods

#### Ethics Statement

All studies performed on mice were in accordance with the Institutional Animal Care and Use Committee protocols in place at Children's Memorial Research Center and specifically approved under protocols 2007-15, 2006-22 and 2008-03.

#### Construction of targeting vectors and generation of Chimeras

The replacement vectors, p(Smn<sup>C-T-Neo</sup>) and p(Smn<sup>2B-Neo</sup>) are shown schematically in Figure 1A. The total length of homology between the targeting vector and the endogenous Smn locus is 6.5 kb; the long arm is 5.0 kb and the short arm is 1.5 kb. The positive selection cassette, flox-pgk-neo was inserted into the unique BamHI site 180 bp distal to exon 7. This was done to increase the...
chance of homologous recombinant clones containing the C-T transition in exon 7 or the 2B mutation (GGA-TTT) in exon 7, since recombination is unlikely to occur within such a short distance between our modification and the positive selection cassette. The mutations were introduced via site directed mutagenesis of a smaller fragment and then re-subcloned into the targeting construct to avoid potential mutations generated by PCR. In total, after Cre excision of the pgk-neo cassette, ~90 bp will remain, of which, 34 bases correspond to the remaining loxP site. The 90 bp of sequence is located sufficiently distal to exon 7 and should not affect regulation or processing of the Smn<sup>C-T-Neo</sup> and Smn<sup>2B-Neo</sup> transcripts. The targeting constructs were electroporated into 129 ES cells and 4 independent homologous recombinant clones for each construct were identified. Two from each construct were used to generate chimeras by microinjecting ES cells into C57Bl/6 blastocysts.

Mice and Animal Care

Animals were kept in a controlled vivarium at 25°C and 50% humidity in a 12 hour light/12 hour dark photoperiod and monitored for health. Colonies were maintained by breeding mice heterozygous for the Smn mutant alleles. The official name for Smn<sup>C-T-Neo</sup>/+ mice is Smn<sup>neo</sup>Cdi. It is being placed at The Jackson laboratory in both C57BL/6 and FVB/N genetic backgrounds but currently lacks official strain designation. The official name of the Smn<sup>2B-Neo</sup>/+ mice is Smn<sup>neo</sup>Cdi and it is also being placed at The Jackson Laboratory in both C57Bl/6 (Jax strain 008838) and FVB/N (JAX 008837) genetic backgrounds. The lines will be available once they are on congenic C57Bl/6 and FVB/N genetic backgrounds.

Genotyping reactions

All PCR and RT-PCR experiments were performed on an Eppendorf Mastercycler®, Smn<sup>C-T-Neo</sup>/+ and Smn<sup>2B-Neo</sup>/+ adults (Table 1) were genotyped using forward primer #638 (5’-AATGTG TGC GAGGCCAGAGG-3’) found within the PGK promoter and reverse primer #637 (5’-TTTGGCAGACTT TAGCAGGGC-3’). Reaction conditions: 94°C/45 sec, 35 cycles. C-T-Neo or 2B-Neo alleles produced a 519 bp product. A 519 bp and 700 bp for the WT allele.

RNA Transcription Analysis

RNA was extracted from tissues using TRIzol reagent (Invitrogen) in accordance with the manufacturer’s directions. Samples were then treated with TURBO DNA-free reagents (Ambion) and first strand cDNA was prepared using SuperScript™ III Reverse Transcriptase (Invitrogen) and random hexamer primers (Roche, Nutley, NJ) according to the manufacturer.

The RT-PCR reaction to amplify both full length Smn and Δ7smn mRNA used primers 435 and 495. All reactions used 100 ng cDNA in a mix of 10X buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 200 μM dNTP mix, 3 mM MgCl₂, 7.5pM primers, and 1U taq polymerase. RT-PCR conditions were as follows: 94°C/45 sec, 60°C/45 sec, 72°C/45 sec, 30 cycles. RT-PCR reaction used to identify the full length products of homozygous mutant embryos (Fig. 3d) was performed using an exon 5 forward primer #741 (5’- TCCCTTTGAGGACCAGCAAATA) and an exon 8 reverse primer #495. Reaction conditions were: 94°C/45 sec, 60°C/45 sec, 72°C/45 sec, 35 cycles. FL-Smn transcripts from MEFs that were used as a template in sequencing were amplified using primers #741 and #495. Δ7smn transcripts were amplified using exon 5-6 forward primer #490 (5’-CTTCAAGG- GACCCCAATTA TC) and exon 6-8 reverse primer, #500 (5’-GACAGAGGCTGAACATA). Both were sequenced with primer #490.

qRT-PCR was performed on cDNA prepared as described above. All reactions were performed in triplicate in a 20 ul final reaction volume. Since the assay on demand primer and probe available from ABI for Smn exon 7 are too close or overlap the C-T and 2B mutations and could hinder interpretation of results, we designed and validated FL-Smn and Δ7smn assays to specifically amplify Smn transcripts with similar efficiencies between the WT, C-T and 2B mutations and not cross react with human SMN. Once validated these assays were prepared by ABI in a 20X mastermix to be similar to an assay on demand. FL-Smn was amplified and detected with primer FL-Smn #ABI-1, Smn678F (5’-TGGCTACACCTGCTGCTAC TATAG), primer #ABI-2, Smn678R (5’-GACCCCAATCTCCTGAGACA) and probe ABI-3, Smn678M (6FAM-CATA- AAAATTAAGAAGTTCAGC). Δ7smn transcripts were amplified and detected with primer #ABI-4, Smn_EX6_6F (5’-GCCAG-TAATGCTACTCTCTGTGTTACA), primer #ABI-5, Smn_EX6_8R (5’-CGACACCCCATCTCTCTGAGA) and probe #ABI-6, Smn_EX6_8M (5’-6FAM-CAGAGGCTGAACATA A T T A T G C, 1 cycle: 10 min 95°C, 40 cycles: 15 sec 95°C, 1 min 60°C. All data was analyzed using the ΔΔCt method.

Protein isolation, western blot and densitometry

Tissues and embryos were homogenized as previously described [55]. Primary antibodies used were mouse monoclonal antibodies to Smn (BD Transabs) at 1:5,000 and β-tubulin at 1:20,000. Polyclonal rabbit actin at 1:1,000. The secondary antibody, goat anti-mouse (BioRad) or goat anti-rabbit (BioRad) were used at 1:10,000. Membranes were exposed to chemiluminescence (ECL Western Blotting Detection Reagents, Amersham Biosciences) and developed on Kodak film. Blots were imported into Adobe Photoshop using Microtek 1000XL scanner and quantified.
through densitometry by Openlab 5.0 software. The Li-Cor Odyssey System was used to quantify embryo extracts in Figure 3 according to the instructions of the manufacturer (Li-Cor Biosciences). Briefly, proteins were resolved on a 4–12% Bis Tris NuPage pre-cast gel system using MES running buffer and transferred to nitrocellulose using the I-Blot transfer system (Invitrogen). Bound antibodies were detected using IR-Dye800CW-conjugated goat anti-mouse IgG (Li-Cor). The intensity of each band was measured and normalized to that obtained from Tubulin.

Whole Mount Embryo Images
Pregnant female mice were sacrificed by CO2 asphyxiation followed with cervical dislocation. Embryos at either E9.5, E12.5 or E13.5 were captured on a Leica upright dissecting microscope and digital camera at 2.0X, 1.0X, and 0.8X magnifications respectively. Embryos at E11.5 were captured at 1.0X. Photographs were taken at a 300dpi, 8 bit/channel quality.

pgk-neo excision by TM in TM in MEFs
Pregnant females were sacrificed with CO2 asphyxiation at 14.5 days post coitum (dpc). Embryos were individually eviscerated and rinsed in 1X PBS for MEF preparation. Yolk sac was collected for DNA to identify Smn<sup>C-T;Neo<sup>−/+;</sup>Cre<sup>E01</sup></sup> embryos. Embryos were finely minced with a straight edge razor and incubated in trypsin at 37˚C. Debris were removed by resuspending cells in 15cc tube containing cell growing media (Earle’s-alpha-MEM, 10% FBS, 1% pen/strep, 1% non-essential amino acids, 1% L-glutamine) and allowing large debris to collect at the bottom. The supernatant was decanted and cells were passaged 4 times before those with the genotype Smn<sup>C-T;Neo<sup>−/+;</sup>Cre<sup>E01</sup></sup> were used for experiments. TM (1 mM) was added to cultures for 1 hr to induce CRE activity. Control cells received no TM. After 24 hours cells were harvested for DNA and RNA as previously described for tissue samples.

pkg-neo excision by TM in adult and embryonic mice
Injection of TM to both adult and embryo used here, were carried out as previously described [37]. TM was diluted to 10 mg/ml in corn oil. Adults were injected with 9 mg TM/40 g body weight and sacrificed five days later. Tissues harvested included spinal cord, skeletal muscle (gastrocnemius and quadriceps), cortex, cerebellum, heart and kidney. Both DNA and RNA were extracted as previously described. For embryonic induction, pregnant females were injected at 7.5 dpc or 13.5 dpc with 3 mg TM/40 g of body weight. At 18.5 dpc, females were sacrificed with CO2 asphyxiation and embryos dissected individually. From each embryo the lung was harvested for DNA and the remainder flash frozen in liquid nitrogen and stored at −80˚C.

Acknowledgments
We would like to thank members of the laboratory for helpful discussions of this research and critically reading this manuscript.

Author Contributions
Conceived and designed the experiments: SMH CJJD. Performed the experiments: SMH VR AB CJJD. Analyzed the data: SMH RGG CJJD. Contributed reagents/materials/analysis tools: CJJD. Wrote the paper: SMH RGG CJJD.

References
1. Schrank B, Goez R, Gunnesen JM, Ure JM, Toyka KV, et al. (1997) Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. Proc Natl Acad Sci U S A 94: 9920–9925.
2. Lefebvre S, Burglen L, Reboullet S, Clermont O, Burlet P, et al. (1995) Identification and characterization of a spinal muscular atrophy determining gene. Cell 80: 155–165.
3. Roberts DF, Chavez J, Court SD (1970) The genetic component in child mortality. Arch Dis Child 45: 35–38.
4. Muntau TL, Davies KE (1992) International SMA consortium meeting. (26–28 June 1992, Bonn, Germany). Neuromuscular Jour 2: 423–428.
5. Wang CH, Finkel RS, Bertini ES, Schroth M, Simonds A, et al. (2007) Consensus statement for standard of care in spinal muscular atrophy. J Child Neurol 22: 1027–1040.
6. Wirth B (2000) An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy. Hum Mutat 15: 228–237.
7. Alias I, Bernal S, Fuentes-Prior P, Barcelo MJ, Albo E, et al. (2009) Mutation update of spinal muscular atrophy in Spain: molecular characterization of 745 unrelated patients and identification of four novel mutations in the SMN1 gene. Hum Genet 125: 29–39.
8. Gennarelli M, Lucarelli M, Capon F, Pizzuti A, Merlina L, et al. (1995) Survival motor neuron gene transcript analysis in muscles from spinal muscular atrophy patients. Biochem Biophys Res Commun 213: 342–348.
9. Monani UR, Lorson CL, Parsons DW, Prior TW, Androphy EJ, et al. (1999) A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci U S A 96: 6307–6313.
10. Lorson CL, Halinen E, Androphy EJ, Wirth B (1999) A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc Natl Acad Sci U S A 96: 6307–6311.
11. Carugo I, Kramer AR (2002) Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. Nat Genet 30: 377–384.
12. Kawasaki T, Manley JL (2003) A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. Nat Genet 34: 460–463.
13. Feldkott M, Schwarzer V, Wirth R, Wiesner TF, Wirth B (2002) Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. Am J Hum Genet 70: 358–368.
27. Heier CR, Satta R, Lutz C, DiDonato CJ (2010) Arrhythmia and cardiac defects are a feature of spinal muscular atrophy model mice. Hum Mol Genet 19: 3906–18.

28. Shahabi M, Habibi J, Yang HT, Vale SM, Sewell WA, et al. (2010) Cardiac defects contribute to the pathology of spinal muscular atrophy models. Hum Mol Genet 19: 4059–4071.

29. Narver HL, Kong I, Burnett BG, Choe DW, Bosch-Marce M, et al. (2006) Sustained improvement of spinal muscular atrophy mice treated with trichostatin A plus nutrition. Ann Neurol 64: 465–470.

30. DiDonato CJ, Chen XN, Noya D, Koresnberg JR, Nadeau JH, et al. (1997) Cloning, characterization, and copy number of the murine survival motor neuron gene: homolog of the spinal muscular atrophy-determining gene. Genome Res 7: 339–352.

31. Viollet L, Bertrand S, Bueno Brunialti AL, Leebre P, Burlet P, et al. (1997) cDNA isolation, expression, and chromosomal localization of the mouse survival motor neuron gene (Snm). Genomics 40: 185–181.

32. DiDonato CJ, Lorson CL, De Repenning Y, Simard L, Chartrand C, et al. (2001) Regulation of murine survival motor neuron (Snm) protein levels by modifying Smn exon 7 splicing. Hum Mol Genet 10: 2727–2736.

33. Hofmann Y, Lorson CL, Stamms S, Androphy EJ, Wirth B (2000) Htra2-beta 1 stimulates an exon splicing enhancer and can restore full-length SMN expression to survival motor neuron 2 (SMN2). Proc Natl Acad Sci U S A 97: 9618–9623.

34. Frugier T, Tiziano FD, Cifuentes-Diaz C, Minion P, Roblot N, et al. (2000) Nuclear targeting defect of SMN lacking the C-terminus in a mouse model of spinal muscular atrophy. Hum Mol Genet 9: 849–858.

35. Hsieh-Li HM, Chang JG, Yong YJ, Wu MH, Wang NM, et al. (2000) A mouse model for activation and inactivation of the mouse Rx homeobox gene. Dev Biol 238: 150–160.

36. Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, et al. (1996) Efficient in vivo manipulation of mouse genomic sequences at the zygote stage. Proc Natl Acad Sci U S A 93: 5860–5865.

37. Hayashi S, McMahon AP (2002) Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol 244: 305–318.

38. Voronina VA, Kozlov S, Mathers PH, Lewandoski M (2005) Conditional alleles for activation and inactivation of the mouse Rx homeobox gene. Genesis 43: 160–164.

39. Lewandoski M (2001) Conditional control of gene expression in the mouse. Nat Rev Genet 2: 743–753.

40. Garcia P, Berlanga O, Watson R, Frampton J (2005) Generation of a conditional allele of the B-myb gene. Genesis 43: 189-195.

41. Levin SL, Meides MH (2004) Floxed allele for conditional inactivation of the voltage-gated sodium channel Scn1a (NaV1.6). Genesis 39: 234–239.

42. Raffai RL, Weissgraber KH (2002) Hypomorphic apolipoprotein E mice: a new model of conditional gene repair to examine apolipoprotein E-mediated metabolism. J Biol Chem 277: 11064–11068.

43. Cifuentes-Diez C, Frugier T, Tiziano FD, Lacene E, Roblot N, et al. (2001) Deletion of murine Smn exon 7 directed to skeletal muscle leads to severe muscular dystrophy. J Cell Biol 152: 1107–1114.

44. Vite JM, Davoult B, Roblot N, Mayer M, Joshi V, et al. (2004) Deletion of murine Smn exon 7 directed to liver leads to severe defect of liver development associated with iron overload. Am J Pathol 165: 1731–1741.

45. Papaioannou VE, Behringer R (2005) Mouse phenotypes: a handbook of mouse mutants. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press. x, 235 p.

46. Meister G, Buhler D, Pillai R, Lotnipesch F, Fischer U (2001) A multiprotein complex mediates the ATP-dependent assembly of spliceosomal U snRNPs. Nat Cell Biol 3: 945–949.

47. Pellizzoni L, Yong J, Dreyfuss G (2002) Essential role for the SMN complex in the specificity of snRNP assembly. Science 298: 1775–1779.

48. Baumer D, Lee S, Nicholoson G, Davies JL, Parkinson NJ, et al. (2009) Alternative splicing events are a late feature of pathology in a mouse model of spinal muscular atrophy. PLoS Genet 5: e1000773.

49. Gabanella F, Butchbach ME, Saieva I, Carissimi C, Burghes AH, et al. (2007) Ribonucleoprotein assembly defects correlate with spinal muscular atrophy severity and preferentially affect a subset of spliceosomal snRNPs. PLoS One 2: e921.

50. Zhang Z, Lotzi F, Dietmar K, Yonnis I, Wan L, et al. (2008) SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. Cell 133: 585–600.

51. Jodelka FM, Ebert AD, Duelli DM, Hastings ML (2010) A feedback loop regulates splicing of the spinal muscular atrophy-modifying gene, SMN2. Hum Mol Genet 19: 4906–17.

52. Schmidt A, DiDonato CJ (2007) Animal models of spinal muscular atrophy. J Child Neurol 22: 1004–1012.

53. Burghes AH, Beattie CE (2009) Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? Nat Rev Neurosci 10: 597–609.

54. Monani UR, Sendmier M, Covett DD, Parsons DW, Andreassi C, et al. (2000) The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn(-/-) mice and results in a mouse with spinal muscular atrophy. Hum Mol Genet 9: 333–339.

55. Heier CR, DiDonato CJ (2009) Translational readthrough by the aminoglycoside genetecin (G418) modulates SMN stability in vitro and improves motor function in SMA mice in vivo. Hum Mol Genet.