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# Disruption of the Survival Motor Neuron (*SMN*) gene in pigs using ssDNA

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## Abstract

Spinal Muscular Atrophy (SMA) is an autosomal recessive neurodegenerative disease that is a result of a deletion or mutation of the *SMN1* (*Survival Motor Neuron*) gene. A duplicated and nearly identical copy, *SMN2*, serves as a disease modifier as increasing *SMN2* copy number decreases the severity of the disease. Currently many therapeutic approaches for SMA are being

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developed. Therapeutic strategies aim to modulate splicing of *SMN2*-derived transcripts, increase *SMN2* gene expression, increase neuroprotection of motor neurons, stabilize the SMN protein, replace the *SMN1* gene and reconstitute the motor neuron population. It is our goal to develop a pig animal model of SMA for the development and testing of therapeutics and evaluation of toxicology. In the development of a SMA pig model, it was important to demonstrate that the human *SMN2* gene would splice appropriately as the model would be based on the presence of the human *SMN2* transgene. In this manuscript, we show splicing of the human *SMN1* and *SMN2* mini-genes in porcine cells is consistent with splicing in human cells, and we report the first genetic knockout of a gene responsible for a neurodegenerative disease in a large animal model using gene targeting with single-stranded DNA and somatic cell nuclear transfer.

### Keywords

Transgenic pigs; Spinal Muscular Atrophy (SMA); Neurodegenerative disease; Animal model; For gene therapy

## Introduction

Spinal Muscular Atrophy (SMA) is the second most common autosomal recessive disorder in humans and is the leading genetic cause of infantile death; however, no cure currently exists. The carrier frequency is approximately 1:35 with an incidence of 1 in 6,000–10,000 live births. SMA is characterized by the progressive degeneration of spinal motor neurons and muscle atrophy. Due to the broad range in motor function, SMA is defined by five clinical types (Type O: severe/birth, Type I: severe; Type II: intermediate; Type III: adolescent/mild; Type IV: adult onset/mild) that are based upon age of disease onset, severity of the symptoms and motor ability (Wirth 2000; Elsheikh et al. 2009; Prior 2010; Prior et al. 2009).

The gene responsible for SMA is called *Survival Motor Neuron-1 (SMN1)*. A nearly identical gene, *SMN2* is present only in humans; however, mutations in *SMN2* have no clinical consequence if *SMN1* is retained. *SMN2* cannot prevent disease development in the absence of *SMN1* but *SMN2* does modify disease severity. *SMN1* and *SMN2* are distinguished in sequence by a few non-polymorphic nucleotide differences that do not alter the coding capacity of *SMN2*. However, a single C to T transition results in the disruption of an exonic splice enhancer (ESE) in exon 7 of *SMN2* (Lorson et al. 1999; Monani et al. 1999; Cartegni and Krainer 2002). As a result, the majority of *SMN2* transcripts are alternatively spliced, resulting in a truncated protein that lacks the 16 amino acids derived from *SMN* exon 7 (Δ7); only approximately ten percent of *SMN2* transcripts are full-length. *SMN*Δ7 protein is unstable and rapidly degraded. *SMN1* alleles almost exclusively produce full-length *SMN* transcripts. All SMA patients retain one or more copies of *SMN2* and there is an inverse correlation between SMA severity and *SMN2* copy number. The relationship between disease severity and *SMN2* is associated with the increase in full-length SMN produced by each additional *SMN2* gene.

Several mouse models have been developed to study SMN function and SMA disease pathology. Deletion of *Smn* in mice, as in all mammals but humans, results in embryonic

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lethality; however, the lethality can be rescued by the introduction of the human *SMN2* transgene (Schrank et al. 1997; Hsieh-Li et al. 2000; Monani et al. 2000). Increasing *SMN2* copy number decreases the disease severity such that mice with two *SMN2* copies have a severe and very progressive phenotype with death within 6 days while mice with eight copies of *SMN2* show no disease phenotype. The addition of *SMN* Δ7, the major splice product of *SMN2*, in a *Smn*−/−;*SMN2* background increases survival to approximately 13 days and demonstrates that the Δ7 product is not detrimental to survival (Le et al. 2005). Mice with a mild SMA phenotype and a lifespan of over 15 months were generated using the *SMN1* (A2G) mutation in a *Smn*−/−;*SMN2* background with a single copy of *SMN2* (Monani et al. 2003). Recently, a new mouse model *Smn*−/−;*SMN2* with three copies of *SMN2* in a C57BL/6 N background produces a slightly less progressive disease phenotype with neuromuscular and breathing defects and a median survival of 14 days (Michaud et al. 2010). While SMA mouse models have proven quite valuable in our understanding of SMN function and SMA, mouse models of SMA have limitations in testing the efficacy of therapeutics. The rapid and progressive post-natal phenotype observed in *Smn*−/−;*SMN2* mice make them in general too severe for therapeutic delivery. As a result, most therapeutic studies to date utilize the *SMN*Δ*7* SMA mice. While the lifespan is slightly longer in *SMN*Δ7 mice, the translation of therapeutics towards clinical application has been difficult.

We have chosen to generate a pig animal model for SMA specifically for the purpose of evaluating the efficacy of SMA therapeutics and for studying their toxicology. We believe a pig SMA model will provide an animal model that closely models humans in size, physiology, metabolism, cardiac output, lung capacity, immune system and nervous system to identify the most efficacious therapeutics. It is anticipated that since pigs have many biological and physiological similarities to the developing human that a pig SMA model will more closely mimic the human condition and expand the therapeutic window. Therefore, the delivery of therapeutics and the analysis of potency, expression and outcome of therapeutics over a prolonged period of time can be studied.

Previous studies have shown that expression of pig SMN in cells and tissues is consistent with that observed in human and mouse cells. Pig SMN co-localizes with human SMN in the cytoplasm and to nuclear gems, and transient transfection of pig *SMN1* into 3813 SMA type I fibroblasts rescues the deficiencies of total SMN protein and gem formation (Lorson et al. 2008). Pig SMN can also rescue deficiencies in snRNP assembly in SMA patient fibroblast extracts (unpublished data). Here we report the production of *SMN*+/− pigs using gene targeting of single-stranded *SMN* DNA and somatic cell nuclear transfer.

## Materials and methods

### RT–PCR assays

Subconfluent human HeLa or pig PK15 (kidney) cells were transfected with 5 μg of the human *SMN1*, human *SMN2*, or the human *SMN1*-Δ*SE2* mini genes using PEI (poylethylenimine). Cells were harvested 24 h post-transfection. Cells were then subjected to a TRIzol (Invitrogen) extraction and RNA was isolated. First-strand cDNA synthesis was performed by using Super Script III (Invitrogen) at 42°C for 50 min followed by 70°C for 10 min. Two microliters of the first-strand synthesis was used in the subsequent PCR

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amplification [94°C for 5 min, (94°C for 45 s, 60°C for 1 min, 68°C for 2 min) × 30] using Vent exo-(Invitrogen). pCI-*SMN1* and pCI-*SMN2* cDNAs served as controls for full-length *SMN* and *SMN*Δ7 respectively. The plasmid-specific primers used were pCI Reverse 5′-agctcgtctgtactattctatgtaa and pCI Forward2 5′-cactataggctagcctcgagaat.

## Targeting vector construction

Genomic DNA was isolated from pig fetal fibroblasts. An 8,640 bp PCR product encompassing *SMN* exons 2a through exon 6 was amplified using LA Taq (Takara). The PCR product was cloned into pCR-Topo TA (Invitrogen) and verified by sequencing. This plasmid is pML. The pML plasmid served as the template to generate the *SMN* recombination arms. pKW2 is a synthesized promoter trap vector with multiple cloning sites 5′ and 3′ of the loxP (locus of X-ing over) sites. Within the loxP sites is an IRES (internal ribosome entry site) sequence, $neo^R$ cassette and SV40 poly A. To build pML8, a 2,538 bp *Acc65*I/*BsrG*I restriction fragment was isolated and cloned into pKW4 to generate the 5′ recombination arm upstream of the $neo^R$ cassette. A 3,468 bp *Sac*I/*Not*I restriction fragment was isolated and cloned into pKW4 to generate the 3′ recombination arm downstream of the $neo^R$ cassette. pKW4 is a synthesized vector containing a $neo^R$ cassette (based on mammalian codon usage) driven by the *pgk* (phosophoglycerate kinase) promoter. LoxP sites flank the *pgk* promoter and $neo^R$ cassette. The sequence junctions of pML8 were verified by sequencing. pML8 was linearized with *Pme*I for electroporation. To generate single-stranded DNA the linearized template was boiled for 5 min and placed on ice.

## Electroporation and selection

Pig fetal fibroblasts were resuspended at a concentration of $1 \times 10^6$ cells/ml in electroporation media (25% optimem +75% cytosalts (120 mM KCl; 15 mM $CaCl_2$; 10 mM $KPO_4$; 5 mM $MgCl_2$) (Ross et al. 2010). Four hundred µl of the cell suspension and linearized pML8 DNA were placed in an electroporation cuvette (4 mM) and the cells were electroporated using 490 volts × 3 pulses for 1 ms/ pulse. The cells were immediately plated to forty 100 mm tissue culture dishes. Thirty-six hours after plating cells were selected using G418 (400 mg/liter) for 11 days. Colonies were harvested using cloning cylinders and screened by PCR.

## PCR screening

Cells were placed in 96-well PCR plates, spun, resuspended in 5 µl of embryo lysis buffer (ELB) (40 mM Tris, pH 8.9; 0.9% Triton X-100; 0.9% Nonidet P-40; 0.4 mg/ml proteinase K) and incubated at 65°C for 15 min and then 95°C for 10 min. For 5′ PCR analysis, fragments were amplified using LA Taq (Takara) and 1 µl of cell lysate using the following parameters: 30 cycles of 30 s at 94°C, 30 s at 60°C and 4 min increasing 10 s/cycle at 68°C with a final extension of 8 min at 72°C. The 5′ PCR primers were: 360L 5′-gcaggtaggcttcttgtggttt 6632R 5′-aagacctgct cctggcatagg. For 3′ PCR analysis, fragments were amplified using LA Taq (Takara) and 1 µl of cell lysate using the following parameters: 30 cycles of 15 s at 94 °C, 30 s at 60°C and 8 min increasing 10 s/cycle at 68°C with a final extension of 10 min at 72°C. The 3′ PCR primers were: 1610L 5′-ggcccgaagatatttccacagt 460R 5′-tgcagaacccatggatacagag. For LR-PCR analysis: LA Taq

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(Takara) and 1.25 μl of cell lysate using the following parameters: 30 cycles of 15 s at 94°C, 30 s at 59°C and 10 min increasing 5 s/cycle at 68°C with a final extension of 10 min at 72°C. The LR-PCR primers were: 360L 5′-gcaggtaggcttcttgtggttt 460R 5′-tgcagaacccatggatacagag.

## Southern blot analysis

Piglet tail snips were digested in approximately 1 ml of genomic isolation buffer (50 mM Tris pH8, 100 mM EDTA, 100 mM NaCl, 1% SDS; 0.4 mg/ ml proteinase K) overnight at 55°C. Genomic DNA was isolated following organic extractions of phenol and chloroform and isopropanol precipitations. Ten μg of genomic DNA was digested with *Eco*RI and separated on a 0.8% agarose gel. Following electrophoresis the DNA was transferred to a nylon membrane. A 1,139 bp fragment (5′*Nco*I and 3′ *Not*I) containing the $neo^R$ cDNA from the KW4 plasmid served as the template to generate a $^{32}$P-αdATP random prime labeled probe. The membrane was prehybridized in Church's Buffer (0.5 M $NaP0_4$, 7% SDS, 10 mM EDTA) at 65°C for 1 h. The membrane was hybridized overnight at 63°C with boiled probe and Church's buffer. The membrane was washed successively with 10× SSC/1% SDS, 2× SSC/1% SDS, 0.5× SSC/1% SDS each for 20 min at 63°C and then exposed to film.

## Nuclear transfer and embryo transfer

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Missouri. Oocytes were purchased from ART Inc. (Madison, Wisconsin). Oocytes were resuspended and matured in maturation medium and cultured for a total of 42–44 h at 38.5°C in 5% $CO_2$ in air. Cumulus cells were removed from oocytes by gentle vortexing in 0.5 mg/ml hyaluronidase. Oocytes were resuspended in micromanipulation medium at 38.5°C. SCNT was performed in micro-manipulation medium supplemented with 7.5 μg/ml cytochalasin B. Metaphase II chromosomes and the polar bodies were aspirated by inserting a micropipette through the zona pellucida. Donor cells were transferred by inserting the pipette into the previously made hole in the zona pellucida and the donor cell was deposited under the zona pellucida. Cells were fused in a low $Ca^{2+}$ solution (0.3 M mannitol, 0.1 mM $CaCl_2.2H_2O$, 0.1 mM $MgCl_2.6H_2O$, and 0.5 mM HEPES), activated with 200 μM thimerosal for 10 min in the dark and incubated with 8 mM DTT for 30 min (Machaty et al. 1997). Oocytes were then washed 3× with PZM3 for 30 min. Fused oocytes were cultured overnight in 500 nM Scriptaid and then washed 3× in PZM3 before being transferred into the recipient (Zhao et al. 2009). The embryonic cleavage rate was determined before transferring the reconstructed embryos into recipients. Recipients on the first day of estrus or the first day after standing estrus were used. Embryo transfer was performed surgically as previously described (Lai and Prather 2003). Recipients were checked for pregnancy by ultrasound. Cesarean section was performed to deliver the piglets on 115–119 days of gestation.

## Quantitative RT–PCR

Fibroblast cells cultured from tail snips of piglets 3-3 and 3-4 were subjected to a TRIzol (Invitrogen) extraction and RNA was isolated. RNA was quantified and one microgram of

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RNA was used for first-strand cDNA synthesis using Super Script III (Invitrogen) and random primers as described by the manufacturer. One microliter of the first-strand synthesis was used in the subsequent PCR amplification. Sequence specific primers to exons 4 and 5 of *SMN* (left primer 5′-cctatgccaggagcaggtct and right primer 5′-agccagcatgacaggaagtg) and *GAPDH* (left primer 5′-actcactcttctacctttgatgct and right primer 5′-tgttgctgtagccaaattca) were used. Three independent first-strand cDNA synthesis reactions were performed for each, JR1 wildtype, piglet 3-3 and piglet 3-4. Each qRT–PCR was performed in triplicate. The PCR reaction was carried out using SYBR green PCR mix (ABI) and the ABI 7500 system. To determine the fold difference between JR1 wildtype and *SMN*+/− piglets the following formulas were calculated. The average of each first-strand cDNA synthesis reaction using *SMN* or *GAPDH* was calculated. The difference between *GAPDH* and *SMN* was determined for each reaction and then the average for all three RT reactions was determined for the JR1 wildtype and *SMN*+/− piglets. Transgenic minus control was calculated with JR1 wildtype at zero and then the fold difference determined. When calculating the fold difference between JR1 (one fold), piglet 3-3 (0.704 fold) and piglet 3-4 (0.996) the variation was not significant when factoring the deviation between piglets of 0.146 fold.

## Results

### Conserved splicing of the human *SMN1* and *SMN2* mini-genes in pig cells

Multiple SMA therapeutic approaches are based on increasing the production of full-length SMN protein from human *SMN2*. Therefore, to produce a successful transgenic pig model of SMA, it was necessary to demonstrate that human *SMN1* and, more importantly, human *SMN2* alternative splicing events are conserved in this animal model.

While *SMN1* homologs exist in all vertebrate species, *SMN2* is unique to humans. Since the human *SMN2* gene will be an important transgene in the development of a pig SMA model, pre-mRNA splicing patterns from previously characterized human mini-genes were examined in the pig kidney cell line PK15 (Fig. 1). *SMN1* and *SMN2* mini-genes correspond to the human genomic region spanning exon 6 through exon 8. The *SMN1*-Δ*SE2* mini-gene carries mutations in the essential splice enhancer SE2 (the hTra2β1 binding element) region and therefore exclusively produces SMNΔ7 transcripts (Lorson et al. 1999). Pig PK15 and human HeLa cells were transfected with human *SMN1*, *SMN2* or *SMN1 exon7*Δ*SE2* mini-genes. Transfected HeLa cells served as positive control for splicing of the mini-genes. The full-length *SMN* and *SMN*Δ*7* cDNAs were used as PCR controls for full-length and exon 7 skipped products, respectively (Fig. 1). The RT–PCR analysis of the mini-genes expressed in PK15 cells and HeLa cells demonstrated similar splicing patterns for each cellular context. Cells transfected with the human *SMN1* mini-gene produced 100% full-length *SMN* product (Fig. 1, lanes 4 and 7). The transfected human *SMN2* mini-gene produced reduced full-length and high levels of the exon7-skipped product in HeLa and PK15 cells (Fig. 1, lanes 5 and 8); while the human *SMN1 exon7*Δ*SE2* mini-gene produced a single exon7-skipped product (Fig. 1, lanes 6 and 9). Amplification of previously cloned *SMN* full-length and *SMN*Δ*7* cDNAs produced the appropriately sized products (Fig. 1, lanes 2 and 3). These results demonstrate that the splicing patterns for the human mini-genes are similar in human

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and pig, providing evidence that the regulation of splicing and splicing factors are conserved and the construction of a porcine SMA model based upon a human *SMN2* transgene would be feasible.

## Development of vectors to target the *SMN* gene in pig fibroblasts

Due to the complex genetics surrounding SMA, the pig model of SMA will be accomplished in three steps. The first step, knockout of the *SMN* allele to produce *SMN*+/− pigs is reported here. The second step, addition of the human *SMN2* transgene, is in progress. The third step is to produce *SMN*−/−;*SMN2* animals through breeding or a second round of nuclear transfer.

We initially chose to target *SMN* using a promoter-trap vector via electroporation, as a promoter-trap strategy was used successfully in targeting the α-1,3-galactosyltransferase gene in pigs (Dai et al. 2002; Lai et al. 2002). A promoter-trap strategy is advantageous as it typically decreases the number of random integration events that give rise to G418-resistant colonies, thereby decreasing the number of false positive G418-resistant colonies. Previous studies demonstrated that *SMN* was robustly expressed in pig fetal fibroblast cells; therefore a *SMN* promoter-trap strategy was possible (Lorson et al. 2008).

We generated a *SMN* promoter trap vector, pML2, with a 5′ recombination arm encompassing exons 2a through 2b and a 3′ recombination arm including part of exon 4 through exon 6. Early passage KW6 and JR1 (Large White, male) fetal fibroblasts were chosen as primary cells based on colony-forming assays (unpublished data). Large White animals were chosen for primary cells due to the larger litter size, piglet weight at birth and that Large White is a maternal breed. Male primary cells were chosen as male pigs can more quickly establish a breeding colony as compared to females.

Fibroblasts were electroporated with linearized, double-stranded pML2 *SMN* DNA. Thirty-six hours following electroporation, cells were selected for 11 days using 0.4 mg/ml G418. G418-resistant colonies were harvested and cell lysates were screened by PCR using a 3-step process (screening the 5′ and then the 3′ recombination arms and then confirming with PCR of the entire targeted region) to reduce the number of false-positive PCR results. In two different fetal fibroblast primary cells (KW6 and JR1) we screened a total of 1,000 G418-resistant colonies and did not identify a *SMN* targeted clone. We postulated that the strength of the *SMN* promoter might not be sufficient to provide significant levels of G418 resistance; therefore, we developed a new *SMN* targeting vector, pML8, which contains the *pgk* promoter driving the $neo^R$ cDNA and flanked by 5′ and 3′ *SMN* targeting arms different than those in pML2 (Fig. 2a). In this vector, the *neo* selection cassette is flanked by *loxP* sites. Homologous recombination of the pML8 *SMN* targeting sequence with the endogenous *SMN* allele results in a 3,467 bp deletion of *SMN* exons 2b, 3 and part of exon 4 with the insertion of the $neo^R$ cDNA between exons 2b and 4. The loss of the Tudor domain, required for U snRNP assembly and Sm protein binding, and the significant deletion and disruption of the SMN reading frame is predicted to result in functional inactivation of the *SMN* gene.

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### Disruption of the pig *SMN* gene by gene targeting using single-stranded *SMN* DNA

Initially JR1 primary cells were electroporated with linearized, double-stranded pML8 DNA. However, after screening more than 500 colonies we did not successfully confirm targeting of the *SMN* locus. We hypothesized and tested whether electroporating single-stranded, linearized pML8 DNA might facilitate homologous recombination and therefore targeting of the *SMN* locus.

G418-resistant colonies were harvested and lysates were screened using the 3-step PCR process. We expected that colonies would be heterozygous for the *SMN* locus with one wild-type allele (endogenous) and one disrupted (targeted) *SMN* allele. All lysates were initially screened using primers (360L-6632R) located outside of the 5′ recombination arm (Fig. 2a). These primers amplify a 5.6 kb endogenous, wildtype *SMN* allele and a smaller 4.8 kb targeted *SMN* allele. All positive lysates were then PCR screened using primers (1610L-460R) located outside of the 3′ recombination arm (Fig. 2a). These primers amplify a 7.5 kb endogenous *SMN* allele and a 6.7 kb targeted *SMN* allele. Lysates positive for targeting both the 5′ and 3′ recombination arms were then confirmed using the long-range PCR (LR-PCR) (Fig. 2a). The LR-PCR amplifies the entire targeted region using primers (360L-460R) outside of the 5′ and 3′ recombination arms. Targeting of the *SMN* locus using LR-PCR is expected to result in a 9.4 kb endogenous product and an 8.6 kb targeted product. Using primers outside of the recombination arms for each PCR was advantageous, as amplification of the endogenous allele served as an internal control for every cell lysate. Additional PCRs using primers (360L-635R and 764L-460R) outside of the 5′ or 3′ recombination arms and within *neo* were used to confirm *SMN* targeting. These PCRs generate single amplimers from the targeted *SMN* allele (Fig. 2a).

Three clones (X8C5, X8D2, Z3A4) were identified and confirmed as positive for *SMN* targeting using primer pairs to the 5′ and 3′ *SMN* targeting arms as well as primers for the LR-PCR and primers to the 5′ and 3′ recombination arms in combination with *neo* primers (Fig. 2b and not shown). The resulting recombination targeting efficiency was 0.9% using linearized, single-stranded pML8 *SMN* DNA. The average targeting efficiency in pigs using electroporation is approximately one to two percent.

Cells from the three positive clones were expanded and frozen. Early passage *SMN*+/− fetal fibroblast cells from clones X8C5, X8D2 and Z3A4 were used for somatic cell nuclear transfer (SCNT). We transferred between 120 and 250 SCNT embryos to each of the 16 recipient females. Six of the recipients of embryo transfer became pregnant. Four of those pregnancies were from clone X8D2 and two were from Z3A4. We did not obtain a pregnancy from X8C5 cells following two embryo transfers. Of those pregnancies, two pregnancies developed to term (~116 days of gestation). Three pregnancies were used for fetal collection and one pregnancy was lost. The first *SMN*+/− piglets, from clone Z3A4, were born on July 19, 2010 (Fig. 3). Of the six piglets born, three piglets survived PND (post natal day) 1. One piglet was undersized and died from acute respiratory distress. Two other piglets were developmentally deficient and were euthanized. The three surviving *SMN* +/− piglets, referred to as 3-2, 3-3 and 3-4 ranged in weight from 0.9 kg to 1.33 kg, grew normally and were weaned. This type of survival rate is not unusual in the generation of

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clones and does not raise any concerns regarding the targeting event related to this specific clone.

### *SMN*+/− offspring

To demonstrate that the Z3A4 piglets were heterozygous at the *SMN* locus we performed PCR using DNA isolated from tail snips using the same primers used to identify the original targeted clones. PCR analysis demonstrates that all six of the offspring delivered on July 19, 2010, were heterozygous for the *SMN* locus with one endogenous wild-type and one targeted allele (Fig. 4).

Genomic DNA was isolated from tail snips of the *SMN*+/− offspring and used for Southern blot analysis. In an attempt to optimize conditions for Southern blot analysis we generated multiple probes but due to the large regions of highly repetitive sequence in *SMN* we did not obtain a *SMN* probe that worked well for Southern blot analysis. As a result, we used sequences specific for *neo* as a probe. Genomic DNA was isolated from tail snips from each piglet and digested with *Eco*RI. A 1.9 kb *Eco*RI band is expected for piglets with a disrupted *SMN* locus due to targeted insertion of the *pgk neo* cassette (Fig. 5a). No bands should be produced from non-targeted, wild-type DNA (JR1). All piglets confirmed as *SMN* targeted by PCR analysis were also confirmed positive for the insertion of *neo* by Southern blot analysis (Fig. 5b). The slightly upshifted band for piglet 3-2 is a result of a slight electrophoresis irregularity on this gel.

To determine whether there was a discernable difference in *SMN* mRNA production in the *SMN*+/−pigs as compared to wild-type pigs, we used quantitative RT–PCR. We did not detect a significant variation in *SMN* RNA abundance between wildtype JR1 and two *SMN*+/− animals (3-3 and 3-4) (Fig. 6). When comparing *SMN* RNA between wildtype (100%), piglet 3-3 (85%) and piglet 3-4 (100%) the percent difference was not substantial when factoring in the deviation between the *SMN*+/− piglets.

## Discussion

Our objective is to generate a large animal model of SMA purposely for evaluating the efficacy of therapeutics. We anticipate that a pig SMA model will more closely recapitulate SMA disease progression in humans, expand the window for therapeutic delivery and allow the analysis of delivery, potency and expression, in order to more efficiently translate bench to bedside.

While a cure for SMA does not currently exist, a number of factors strongly suggest that SMA is well positioned for translational success: (1) The gene deleted in SMA, *SMN1*, has been identified. Mutations and deletions in *SMN1* account for nearly all cases of SMA. (2) The nearly identical copy gene, *SMN2*, is present and functions as a disease modifier. (3) Diagnostic screens are available and are being improved for newborn screening. (4) Advancements in drug development, gene therapy and *SMN2* modulation are progressing toward clinical application (Lorson et al. 2010; MacKenzie 2010; Stavarachi et al. 2010). Reports indicate that compounds can increase either total transcription from *SMN2* and/or exon 7 inclusion (Andreassi et al. 2001; Chang et al. 2001; Zhang et al. 2001; Brichta et al.

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2003; Sumner et al. 2003; Andreassi et al. 2004; Wolstencroft et al. 2005; Mattis et al. 2006; Thurmond et al. 2008; Hastings et al. 2009; Mattis et al. 2009a, b; Butchbach et al. 2010; Riessland et al. 2010). Modulation of *SMN2* splicing patterns using nucleic acid based therapeutics has shown to increase full-length SMN (Lim and Hertel 2001; Madocsai et al. 2005; Baughan et al. 2006; Coady et al. 2008; Baughan et al. 2009; Geib and Hertel 2009; Singh et al. 2009; Coady and Lorson 2010; Lorson et al. 2010; Hau et al. 2008, 2010). Recent gene replacement studies have demonstrated that delivery of AAV8*SMN* or AAV9*SMN* into SMA mice resulted in improvement in muscle function and lifespan (Foust et al. 2010; Passini et al. 2010; Valori et al. 2010; Dominguez et al. 2011). A large animal model of SMA to test these translational approaches would be a very powerful tool towards clinical application. To this end, we report the targeted disruption of the *SMN* gene in pigs.

We have demonstrated through *SMN2* splicing assays that a SMA pig model based on the *SMN2* transgene is feasible and that the therapeutic benefit of compounds and small molecules based on exon 7 inclusion and total full-length *SMN* should be readily assayed using traditional methods. Splicing of *SMN1* and *SMN2* appears to be highly conserved as similar ratios of full-length and exon 7 skipped products are produced in a variety of experimental contexts such as mice, rats, zebrafish and now swine.

Towards a porcine model of SMA we have generated three *SMN*+/− knockout fetal fibroblast clones, X8C5, X8D2 and Z3A4 by homologous recombination of a *SMN* targeting vector. We found that homologous recombination of the pML8 targeting construct was greatly enhanced by electroporation of single-stranded, linearized DNA as compared to double-stranded, linearized DNA from the same construct. One potential explanation is that homologous recombination is more efficient when providing a single-stranded DNA. We are investigating whether providing a single-stranded DNA fragment with homologous sequence on both ends will increase *SMN* gene targeting.

Nuclear transfer of *SMN* targeted clone Z3A4 produced three healthy *SMN*+/− piglets demonstrating that like their human counterparts *SMN*+/− pigs are phenotypically normal. PCR and Southern blot analysis have confirmed that we have targeted the *SMN* locus. While we did not detect a significant reduction in *SMN* RNA between wildtype and *SMN*+/− animals, these results are consistent with the levels of *SMN* RNA being tightly regulated through an autoregulatory feedback mechanism and in keeping with the housekeeping role of SMN in snRNP assembly. Loss of one *SMN* allele is not detrimental as upregulation in expression of the second *SMN* allele can maintain cellular activities. Importantly, these studies remain consistent with knockout of a single *SMN* allele.

An additional nuclear transfer from clone Z3A4 has resulted in a pregnancy and, on November 1, 2010, the birth of six healthy and phenotypically normal *SMN*+/− piglets. All six piglets are healthy to date. *SMN*+/− animals will be grown to sexual maturity and outcrossed to wild-type females to generate *SMN*+/− animals for breeding.

Our goal is to develop SMA pigs to specifically address the need for a large SMA animal model for therapeutic analysis. We have completed the first step towards this goal in generating *SMN*+/− pigs. We are currently using the *SMN*+/− fetal fibroblast cells to

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introduce the human *SMN2* transgene, step two, towards generating SMA *SMN−/−; SMN2* pigs.

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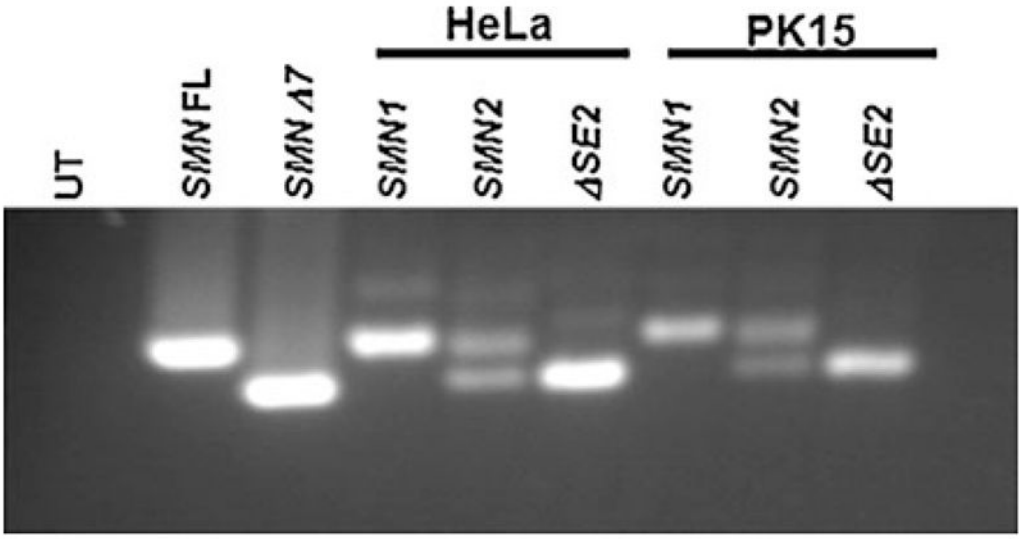


**Fig. 1.**

Splicing of the human *SMN1* and *SMN2* mini-genes. Pig kidney (PK15) and human HeLa cells were transfected with the *SMN1*, *SMN2* or *SMN1*Δ*SE2* mini-genes. The relative ratio of full-length to Δ7 *SMN* RNA was analyzed by RT-PCR. *SMN1* and *SMN*Δ*7* correspond to full-length and exon 7 skipped products, respectively (*lanes 2 and 3*). Cells transfected with the human *SMN1* mini-gene produced exclusively full-length *SMN* product in HeLa and PK15 cells (*lanes 4 and 7*). The transfected human *SMN2* mini-gene produced full-length and exon7-skipped products (*lanes 5 and 8*); while the human *SMN1 exon7*Δ*SE2* mini-gene produced entirely exon7-skipped product (*lanes 6 and 9*) in human and pig cells

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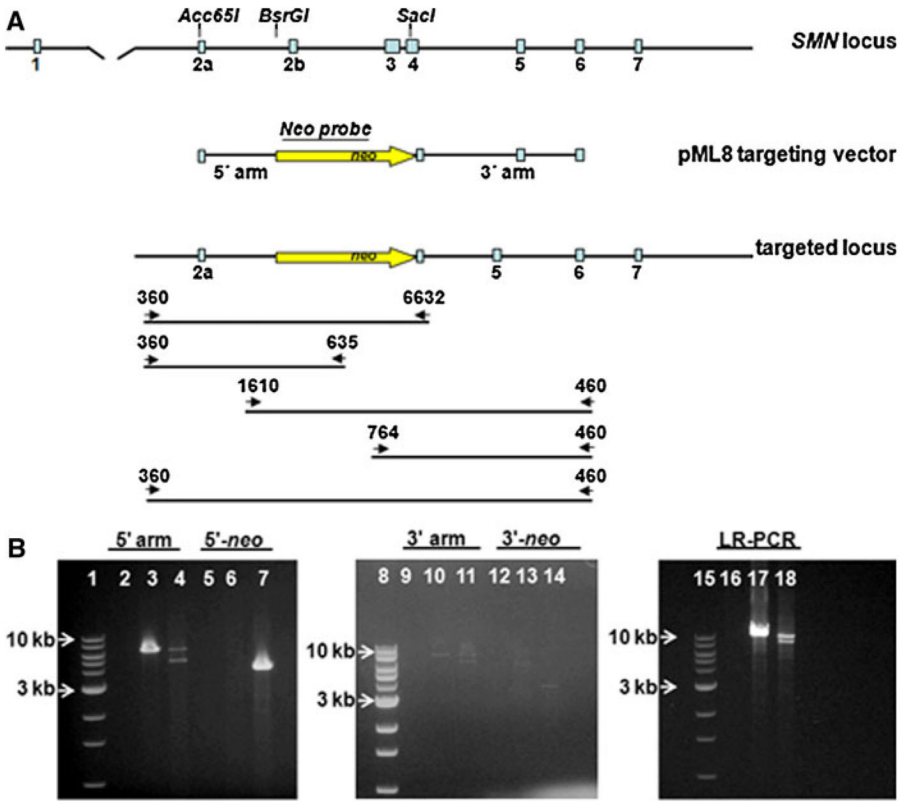


**Fig. 2.**

Disruption of the pig *SMN* locus by gene targeting. **a** Diagram of the pig *SMN* locus, the corresponding pig *SMN* genomic sequence used to construct the 5′ and 3′ recombination arms for the pML8 targeting vector and the targeted locus following homologous recombination with the pML8 targeting sequence. The primers 360L and 6632R were used to screen for recombination of the 5′ arm. The primers 1610L and 460R were used to screen for recombination of the 3′ arm. The primers 360L and 460R were used for long range PCR. Primers 360L and 635*neo*R and 764 *neo*L and 460R were used to screen for the presence of *neo*. **b** PCR of pig fetal fibroblast cells from colony X8D2. *Lanes 1, 8 and 15* are 1 kb DNA ladders. For each primer pair, the templates are no template control, JR1 wildtype and X8D2 pig cell lysates respectively. *Lanes 2–4* are PCRs using primers 360L and 6632R. *Lanes 5–7* are PCRs using primers 360L and 635*neo*R. *Lanes 9–11* are PCRs using primers 1610L and 460R. *Lanes 12–14* are PCRs using primers 764 *neo*L and 460R. *Lanes 16–18* are PCRs using primer pairs 360L and 460R

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**Fig. 3.**

*SMN* gene knockout piglets at 10 days of age. The first *SMN*+/− piglets, from clone Z3A4, were born July, 2010. Of the six piglets born, three piglets survived PND (post natal day) 1. The three surviving *SMN*+/− piglets are 3-2, 3-3 and 3-4

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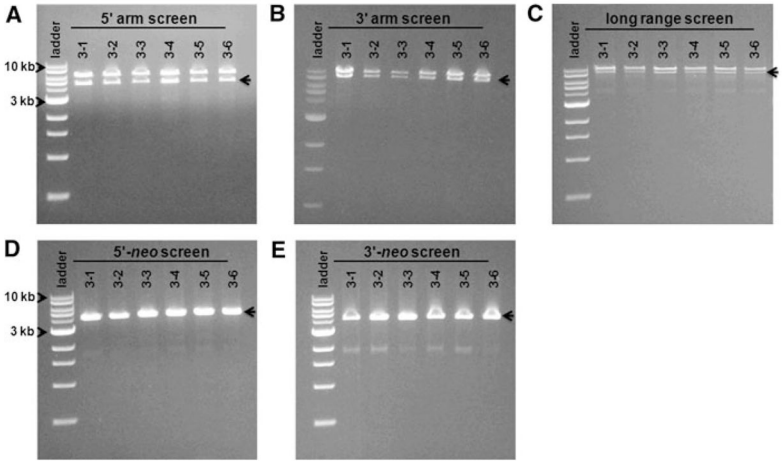


**Fig. 4.**

Piglets generated from colony Z3A4 are heterozygous at the *SMN* locus. DNA isolated from tail snips from piglets 3-1 to 3-6 was used for PCR with the same primers as in Fig. 2. Lane 1 for each set is a 1 kb DNA ladder. **a** PCR demonstrating recombination at the 5′ arm using primers 360L-6632R. A larger 5.6 kb wildtype allele and a smaller 4.8 kb targeted allele were amplified. **b** Recombination at the 3′ arm using primers 1610L-460R. A 7.5 kb wildtype allele and a 6.7 kb targeted allele were amplified. **c** LR-PCR amplification of the *SMN* targeted region. Wildtype allele is 9.4 kb while the *SMN* targeted allele is 8.6 kb. **d, e** Amplification of *SMN* targeted alleles using primers to 5′ and 3′ recombination arms and *neo*, respectively. Negative controls for the primer pairs are in Fig. 2 and not shown. The *arrow* indicates the targeted amplimer

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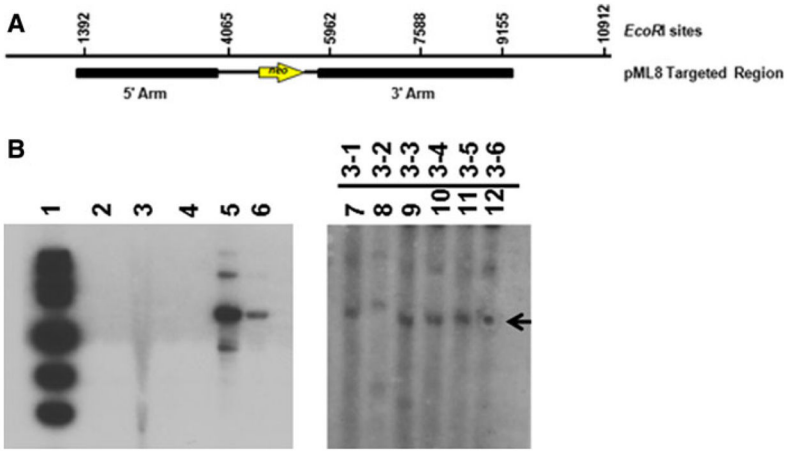


**Fig. 5.**

Southern blot analysis of *SMN*+/− piglets. **a** Diagram depicting the *Eco*RI sites within the *SMN* targeted sequence with the pML8 targeted genomic region as reference. **b** Genomic DNA was isolated from tail snips of piglets 3-1 to 3-6 and digested with *Eco*RI. *Lane 1* is the 1 kb DNA ladder. *Lane 2* is no DNA control. *Lane 3* is wildtype (*SMN*+/+) JR1 genomic DNA digested with *Eco*RI. *Lane 4* is a blank lane. *Lanes 5 and 6* are a *Blp*I digested control plasmid containing *neo* with 10 or 1 molecules of DNA, respectively. *Lanes 7–12* contain genomic DNA from piglets 3-1 to 3-6 digested with *Eco*RI. A 1,139 bp *Nco*I/*Not*IDNA fragment isolated from the KW4 plasmid that contains the $neo^R$ cDNA served as a probe. Exposure for *lanes 1–6* was 1 day. Exposure for *lanes 7–12* was 6 days

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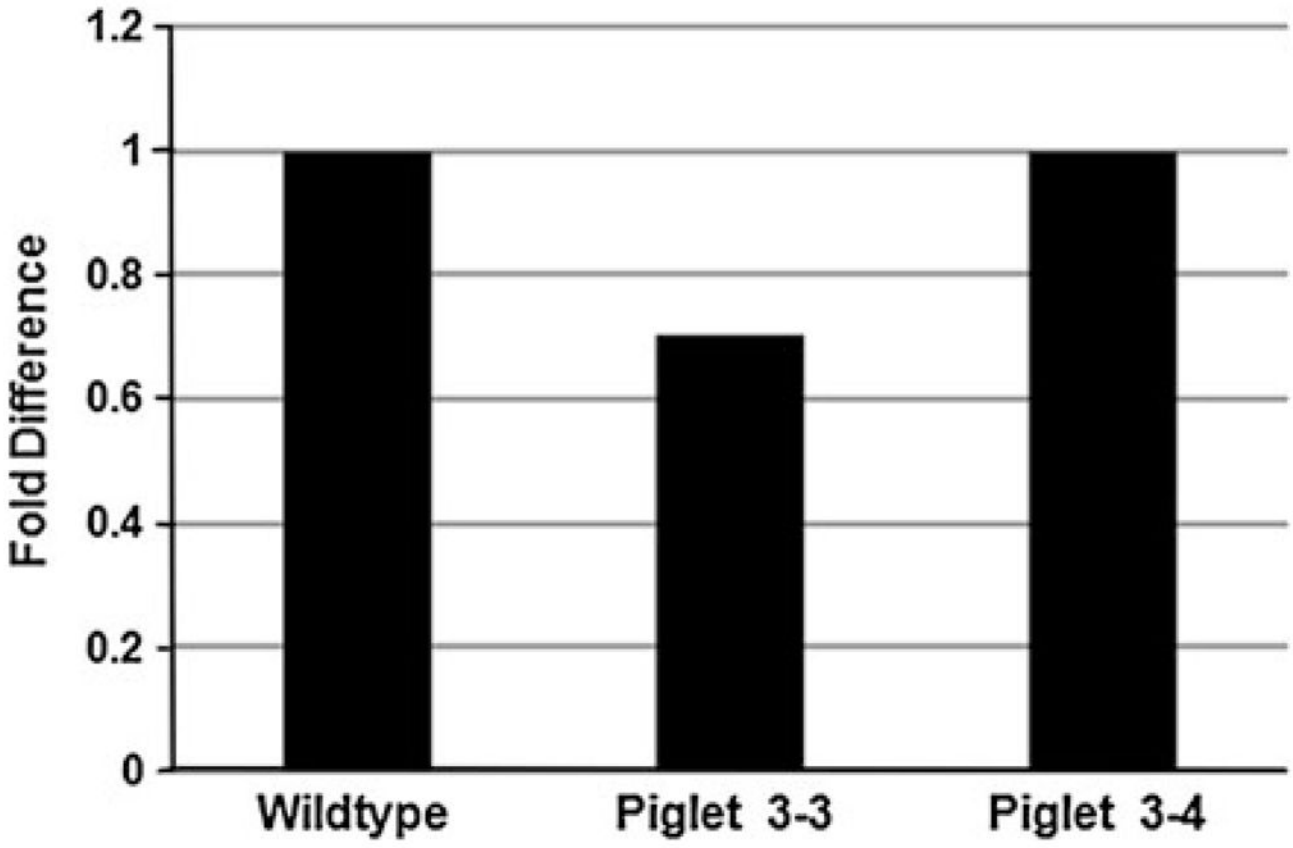


**Fig. 6.**

*SMN* RNA expression in *SMN*+/− piglets. Total RNA was isolated from tail fibroblast cells cultured from JR1 (*SMN*+/+ cells) and piglets 3-3 and 3-4. *SMN* RNA was quantified by qRT-PCR in wildtype and *SMN*+/− cells