Low levels of Survival Motor Neuron protein are sufficient for normal muscle function in the SMNΔ7 mouse model of SMA

Chitra C. Iyer1,†, Vicki L. McGovern1,†, Jason D. Murray2, Sara E. Gombash3, Phillip G. Zaworski6, Kevin D. Foust3, Paul M.L. Janssen2 and Arthur H.M. Burghes1,4,5,*

1Department of Molecular and Cellular Biochemistry, 2Department of Physiology and Cell Biology, 3Department of Neuroscience, 4Department of Neurology, 5Department of Molecular Genetics, The Ohio State University, Columbus, OH 43210, USA and 6PharmOptima, LLC, Portage, MI 49002, USA

*To whom correspondence should be addressed at: Department of Molecular and Cellular Biochemistry, 364 Hamilton Hall, 1645 Neil Avenue, The Ohio State University, Columbus, OH 43210, USA. Tel: +1 6146884759; Fax: +1 6142924118; Email: burghes.1@osu.edu

Abstract

Spinal Muscular Atrophy (SMA) is an autosomal recessive disorder characterized by loss of lower motor neurons. SMA is caused by deletion or mutation of the Survival Motor Neuron 1 (SMN1) gene and retention of the SMN2 gene. The loss of SMN1 results in reduced levels of the SMN protein. SMN levels appear to be particularly important in motor neurons; however SMN levels above that produced by two copies of SMN2 have been suggested to be important in muscle. Studying the spatial requirement of SMN is important in both understanding how SMN deficiency causes SMA and in the development of effective therapies. Using Myf5-Cre, a muscle-specific Cre driver, and the Cre-loxP recombination system, we deleted mouse Smn in the muscle of mice with SMN2 and SMNΔ7 transgenes in the background, thus providing low level of SMN in the muscle. As a reciprocal experiment, we restored normal levels of SMN in the muscle with low SMN levels in all other tissues. We observed that decreasing SMN in the muscle has no phenotypic effect. This was corroborated by muscle physiology studies with twitch force, tetanic and eccentric contraction all being normal. In addition, electrocardiogram and muscle fiber size distribution were also normal. Replacement of Smn in muscle did not rescue SMA mice. Thus the muscle does not appear to require high levels of SMN above what is produced by two copies of SMN2 (and SMNΔ7).

Introduction

Spinal muscular atrophy (SMA) is the leading genetic cause of infant death affecting 1 in approximately 10 000 live births (1,2). SMA is caused by decreased levels of the Survival Motor Neuron (SMN) protein (3,4). Humans have two genes coding for Survival Motor Neuron (SMN), SMN1 and SMN2. SMN1 and SMN2 essentially differ by a single nucleotide C → T change in exon 7 that alters a modulator of splicing such that the majority of SMN transcripts from SMN2 lack exon 7 (5–8). SMNΔ7 protein does not oligomerize efficiently and is rapidly degraded. SMN2 thus produces only a small amount of full-length SMN that is not sufficient for motor neuron survival. SMA is an autosomal recessive disease caused by deletion or mutation of the SMN1 gene and retention of the SMN2 gene (9,10). Based on the clinical severity, SMA is classified into five categories: Type 0–IV, wherein SMA Type 0 is the most severe form of the disease. The severity of SMA shows an inverse correlation to SMN2 copy number (10,11).
SMN protein functions in the assembly of snRNPs, by loading the Sn proteins onto the snRNA (10,12,13). It has also been suggested to act as a master ribonucleoprotein assembler (10,14–16), assembling the Sm-Lsm10/11 ring on the U7snRNA as well as other protein complexes on other RNAs (17–19). It is worth noting, of the many RNA-related roles proposed for SMN, there has been conclusive demonstration only of its activity in canonical snRNPs and U7asnRNP assembly (16). Since SMN is also found in the axons of neurons, it has been proposed that SMN could play a role in assembling the transport granules of axons (20–22). Although SMN is expressed in all tissues, SMA particularly affects motor neurons and results in atrophy of muscle. We have previously shown that high expression of SMN in neurons with low levels in other tissues gives substantial rescue of severe SMA mice (23). In addition we showed that expression of very high levels of SMN in skeletal muscle with no leakage into other organs had no impact on the SMA phenotype. However with high expression of SMN in skeletal and cardiac muscle and a low level of SMN (above that produced by SMN2) in all tissues, the mice survived to 160 days (23). If SMN is removed from muscle but retained in other tissues then a severe muscular dystrophy results (24). However complete loss of SMN is not what occurs in SMA. A minimum amount of SMN is required in all cell types; complete removal of SMN leads to cell death (24–26). Since atrophy of muscle is a symptom of SMA and muscle supports motor neurons by the production of growth factors, treatment of the muscle tissue has been explored. Transgenic expression of follistatin showed a minimal improvement in muscle mass with no improvement of motor function or survival (27). Administration of follistatin, which blocks myostatin, a negative regulator of muscle mass, did not improve the survival of SMA mice (28); neither did transgenic inactivation of murine myostatin in SMA mice (29). Moreover, homozygous knock out of muscle ubiquitin ligases, MAFbx and MuRF1, did not improve the survival or muscle mass of SMA mice (30). However in all cases the improvement in muscle mass was minimal and thus it is still possible, for instance in mild SMA, that increased muscle bulk would be beneficial.

A question that has not been addressed is how muscle would perform when completely reliant on two copies of SMN2 for its SMN protein requirement. Does SMN reduction, not removal, affect the ability of muscle to produce force? In the current work, we address this question directly. To get a complete picture of the role of the muscle in SMA, we not only deleted SMN specifically in the muscle, but also replaced Smn in the muscle. We chose to use the Myf5-Cre driver so as to remove mouse Smn from both myoblasts and myotubes in mice containing two copies of SMN2 and SMN7 transgenes (31,32). We found that decreasing SMN in muscle does not hamper the muscle’s force production or fiber size or morphology. Moreover, the total body weight and survival of mice with decreased SMN in muscle remain unchanged. Conversely, replacement of SMN in muscle tissue of SMA mice with SMN deleted to SMA-levels elsewhere resulted in no improvement in survival or body weight of the SMA mice. Thus we conclude that the muscle tissue per se can function in a completely normal manner when SMN is at reduced levels and that the muscle does not play a crucial role in SMA pathogenesis.

**Results**

**Testing the deletion and replacement Smn alleles**

To selectively reduce SMN in a tissue, the murine line Smn<sup>F7</sup> with floxed exon 7 was used (25) as one allele, with the other allele being the exon 2A disruption, Smn<sup>ko</sup> allele (33), which results in the production of no functional mouse SMN. The floxed Smn lines and the Cre lines were each first crossed on to the SMNΔ7 SMA background and were made homozygous for the SMN2 and SMNΔ7 transgenes and the Cre lines were made heterozygous for Smn<sup>ko</sup>. Thus, SMN2 and SMNΔ7 in the background provide low SMN essential for viability in all cells. Breeders positive for Cre and the Smn<sup>ko</sup> allele (32–34) were bred to the floxed Smn mouse lines. As shown in Figure 1A, upon action of Cre, exon 7 gets deleted, resulting in Smn<sup>ko</sup> allele (for deletion of Smn), creating an SMA-situation in the places Cre is expressed. For replacement experiments, we used the rescue allele Smn<sup>INV</sup> (also referred to as Smn<sup>INV</sup> in other works) (35). Smn<sup>INV</sup> allele is a hybrid allele with human SMN exon 7 joined to inverted mouse exon 7, flanked by lox66 and lox71 sites, which enable reversion of the cassette upon Cre recombination (36). Thus the floxed inverted mouse exon 7 is reverted to the correct orientation upon Cre recombination, called Smn<sup>INV</sup> (for reversion of exon 7), and expression of mouse Smn is restored (Fig. 1A). We sequenced the Smn<sup>INV</sup> and Smn<sup>ko</sup> alleles (Supplementary Material, Fig. S4).

In previous experiments with Cre drivers, the floxed lines, Smn<sup>F7</sup> and Smn<sup>INV</sup>, have been used with both Smn alleles bearing the floxed allele (24–26,35,37–39). We chose to have one allele that could either be deleted or reverted; the design of crosses is shown in Figure 1B. This was for two reasons: first the Cre driver can be separated from the floxed exon 7 allele, thus eliminating any chance of germline conversion, and second, the Cre only has to act on one allele, maximizing its chance of action on the floxed allele. To first demonstrate that the floxed Smn alleles, Smn<sup>F7</sup> and Smn<sup>INV</sup>, for deletion and replacement respectively are fully functional, we crossed the floxed Smn alleles to a ubiquitous Cre line, Sox2-Cre. The Sox2-Cre transgenic line has expression of Cre under the Sox2 promoter element and efficient Cre recombination is seen in all epiblast-derived cells by e6.5 (40). Sox2-Cre mice were crossed onto the SMA background and made homozygous for SMN2 and SMNΔ7. Mice with Sox2-Cre; Smn<sup>F7/KO</sup> have Smn-deletion in all tissues and thus all tissues are dependent on SMN produced by SMN2 and SMNΔ7. Sox2-Cre; Smn<sup>INV/KO</sup> mice had a phenotype and weight (Fig. 1D, n = 9, blue inverted triangle) similar to SMNΔ7 SMA mice and a mean survival of 13.8 ± 1.3 days (Fig. 1C, n = 9, blue line). Upon crossing the Sox2-Cre; Smn<sup>INV</sup>/KO mice to the replacement allele, Smn<sup>INV</sup>, we obtained complete rescue of phenotype (Fig. 1C, n = 10, red line) and weight (Fig. 1D, n = 10, red diamond). The rescue mice, Sox2-Cre; Smn<sup>INV/KO</sup> appeared normal in phenotype, with no necrosis or decrease in weight.

**Expression of Myf5-Cre driver**

We chose Myf5-Cre line to drive Cre in the muscle because Myf5 is the first skeletal muscle marker expressed by e8.5 in immature somites before terminal myogenesis (41,42). Thus Smn will be deleted or replaced in the early stages of skeletal muscle development. Myf5-Cre line was made homozygous for SMN2 and SMNΔ7. To test the expression of Myf5-Cre, we crossed the Cre line to an RFP reporter line, ROSA26-loxP-STOP-loxP-tdTomato (43). The reporter line has a floxed STOP codon upstream of tdTomato, a modified RFP gene. Upon excision of the STOP codon by Cre, RFP is expressed in that tissue (43). To visualize motor neurons, mice with Hb9:GFP which expresses GFP in the motor neurons under the murine Hb9 promoter at e9.5 (44), were crossed to the tdTomato-RFP mice. Figure 2 shows immunos- tained spinal cord and skeletal muscle sections of post-natal day 12 (PND12) mouse pups bearing Myf5-Cre, tdTomato and Hb9:GFP. Robust RFP expression was observed in the skeletal muscle (Fig. 2D and E). However, 1–3 motor neurons in the spinal cord also showed RFP expression in 4/7 animals we studied; 3/7

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Figure 1. Smn-deletion and replacement alleles used in the study. (A) Diagram of mouse Smn alleles used for the Cre recombination experiments: SmnF7 allele contains floxed exon 7 wherein exon 7 is deleted upon Cre recombination, giving the allele, SmnD7 (25). SmnINV allele has mouse Smn exons 1–6 fused to human SMN exon 7 with an inverted mouse Smn exon 7. The lox66 and lox71 sites flanking the fused human and mouse exon 7 construct get reverted upon Cre recombination. With mouse Smn exon 7 in the right orientation, SmnKO produces full-length SMN protein (35). Red arrows indicate the lox sites. (B) Diagrammatic representation of strategy of mouse breeding: To avoid germline recombination and ensuring that Cre acts on only one floxed allele, we chose to maintain the floxed lines and Cre line separately. The Cre lines bore the SmnKO allele. These were crossed to either SmnF7 allele maintained in a homozygous state or SmnINV that was maintained over an SmnWT allele. The affected mice with Smn-deletion thus had SmnD7/KO and the Smn-replacement mice had SmnINV/KO. (C) Testing the floxed alleles with the ubiquitous Sox2-Cre driver: Deletion (blue line): Sox2-Cre, SmnD7/KO mice (n = 9) had a mean survival of 13.8 ± 1.3 days which is similar to SMA mice (14.3 ± 0.7 days, n = 16, green line). Replacement (red line): Sox2-Cre, SmnINV/KO mice (n = 10) were phenotypically normal and survived beyond 36 weeks. (D) The total body weights of Sox2-Cre, SmnD7/KO mice (n = 9, blue inverted triangle) are similar to SMA mice (n = 16, green circle) while the weights of Sox2-Cre, SmnINV/KO mice (n = 10, red diamond) are similar to controls (Sox2-Cre, SmnINV/WT). Thus, the SmnF7 and SmnINV alleles are functional. (Error bars = sem).
animals showed no RFP expression in the motor neurons (Fig. 2A-C). Out of four Myf5-Cre; tdTomato; HB9::GFP animals examined, leaky expression was found in some cells in the heart, lung, liver and pancreas in half of the animals examined. Only one animal showed RFP expression in kidney and adrenal gland (Supplementary Material, Fig. S1). Scattered blood vessels and connective tissue cells were also positive for Myf5-Cre expression in all organs examined above. In all cases the leaky expression was in a few cells in the entire section of the tissue. Also, it should be noted that not all animals showed leaky expression in every tissue and to the same degree. Myf5-Cre is thus a chimeric driver with ectopic expression in a small percentage of cells in other tissues. Unlike the tissues mentioned above, the skeletal muscle consistently showed uniform strong expression of RFP. We thus used the Myf5-Cre line to determine the importance of SMN levels above that produced by two copies of SMN2 (and SMNδ7) to the normal function of muscle.

Deletion and replacement of Smn in muscle tissue in the SMNδ7 SMA mouse model

We selectively reduced SMN levels in the muscle using the Myf5-Cre driver (41). The Myf5-Cre; SmnΔ7/KO, Smn2+/−; SMNδ7+/− mice had deletion of the exon 7 of the floxed Smn allele in the skeletal muscle. Thus the muscle becomes reliant on the SMN produced by the SMN2 (and SMNδ7) that is replaced in the muscle tissue in the SMNδ7 SMA mouse model.

Efficiency of Cre recombination in the muscle

To determine the efficiency of Cre excision with a more direct read-out, we quantified the percentage of Cre recombination by performing droplet digital PCR (ddPCR, BioRad) on the SmnΔ7 allele in the muscle tissue. Importantly, we eliminated the influence of connective tissue by laser-capture microdissection (LCM) of gastrocnemius muscle cells, leaving out the nuclei of extrasarcomeric tissue (n = 3 per genotypic group). ddPCR of SmnΔ7 was normalized to Smn intron 1 (two copies per genome) in a multiplex reaction. ddPCR of LCM-collected Sox2-Cre; SmnΔ7/KO gastrocnemius muscle (PND9–15) showed 100 ± 5.5% recombination while No Cre; SmnΔ7/KO had no recombination (0.6 ± 14.7%). Myf5-Cre; SmnΔ7/KO gastrocnemius (PND10) tissue collected via LCM showed 80.8 ± 4.2% recombination (Fig. 4A), thus proving that Myf5-Cre efficiently acts on the floxed alleles in the skeletal muscle. The efficiency of Cre recombination by Myf5-Cre is less than the expected cent percent for two reasons as follows. Myf5-Cre is not expressed in the subsynaptic muscle nuclei (Dr Jill Rafael-Fortney, personal communication). In our immunohistochemistry studies, we have observed specks of HB9::GFP staining indicative of the presence of motor neuron in muscle sections (data not shown); implying that the muscle cell adjoining the motor neuron would be a subsynaptic muscle cell not expressing Myf5-Cre. Secondly, because the technique of LCM aids in the
collection of a muscle nuclei-enriched sample, we cannot rule out the possibility that our samples would have a small percentage of connective tissue that do not express Myf5-Cre. Thus the presence of nuclei of subsynaptic muscle cells and connective tissue would cause the efficiency of Myf5-Cre recombination to be reported as less than 100 percent in our experiment. Hence an efficiency of 80% Myf5-Cre recombination is sufficient to imply complete recombination of floxed Smn alleles in the muscle cells.

Figure 3. Survival and weight analyses of muscle driver Myf5-Cre: (A) Deletion (blue line): Affected mice, Myf5-Cre; SmnF7/KO (n = 15), had a normal survival. Replacement (red line): Mean survival of affected mice, Myf5-Cre; SmnF7/KO mice (n = 13) was 14.1 ± 2.4 days, similar to SMA mice (14.3 ± 0.7 days, n = 16, green line). (B) In Myf5-Cre; SmnD7/KO mice, the weight curve was similar to controls (blue inverted triangle, P > 0.05 versus controls). Mice with Smn-replacement in the muscle, Myf5-Cre; SmnF7/KO (n = 11, red diamond) were similar to SMA mice (n = 16, green circle) in body weight (P > 0.05 versus SMA) (Error bars = sem).

Figure 4. Analysis of Cre activity upon deletion of Smn in muscle. (A) Quantification of percentage of Cre recombination on the SmnF7 allele: Digital droplet PCR (ddPCR) of muscle nuclei collected via LCM of H&E stained gastrocnemius muscle of PND9-15 mice show complete recombination by the Sox2-Cre driver (100 ± 5.5%). The Myf5-Cre driver specific for skeletal muscle showed 80.8 ± 4.2% recombination of SmnF7 allele. (B) ELISA-estimation of SMN protein levels in whole muscle sections (PND10): Smn-deletion with Myf5-Cre leads to a drop in muscle SMN levels to 1510.9 ± 529.8 pg SMN/mg soluble protein from 4013.8 ± 1721.9 pg in No Cre; SmnF7/KO mice. SMA mice had a fall in SMN levels to 160.8 ± 33.8 pg/mg of soluble protein. (C) Representative western blot bands of SMN and tubulin from No Cre; SmnF7/KO and Myf5-Cre; SmnD7/KO mice. (D) SMN protein levels determined by western blot relative to tubulin (PND10): A 68.7% decrease in SMN was observed in whole muscle of Myf5-Cre; SmnD7/KO mice. SMN to tubulin ratios at 2.08 ± 0.13 in No Cre; SmnF7/KO control were significantly different from 0.65 ± 0.26 in affected mice (P = 0.009) (n = 3 in each group, error bars = sem).
Determination of SMN protein levels in muscle

We quantified SMN protein via ELISA immunoassay (proprietary, PharmaOptima, MI, USA) in cryostat-sectioned whole muscle of PND10 pups. Myf5-Cre; Smn<sup>D7/KO</sup> mice, as shown in Figure 4B, showed a decrease in SMN levels. The SMN level in the Myf5-Cre mice was higher than the SMA sample, which is not unexpected due to SMN contribution from non-muscle cells such as connective tissue and blood vessels. There was no statistical difference between SMN levels in SMA and Myf5-Cre; Smn<sup>D7/KO</sup> mice as the ELISA assay showed relatively high variation. We thus also analyzed SMN protein levels in skeletal muscle via western blotting. Figure 4C and D respectively show representative bands of SMN and tubulin with quantitation of No-Cre; Smn<sup>F7/KO</sup> and Myf5-Cre; Smn<sup>D7/KO</sup> mice (PND10, n = 3 in each group). Upon deletion of Smn with Myf5-Cre, there was a significant decrease of SMN protein in skeletal muscle to 31.3% of control No-Cre levels (2.08 ± 0.13 versus 0.65 ± 0.26, P = 0.009). We believe an approximate drop of 70% in SMN levels is sufficient to imply good recombination by Myf5-Cre in muscle. In addition to small amounts of SMN protein produced by SMN2 and SMNA7, there would be contribution of non-muscle cells of connective tissue and post-synaptic muscle as described above in the whole muscle lysate. Martinez et al. reported a robust increase of SMN transcript and protein upon replacement of Smn using Myf5-Cre (37).

Other groups have shown efficient recombination by Myf5-Cre in muscle and an 80% drop in RNA and protein levels of the floxed allele (45). Beedle et al. have shown that deletion of Fukutin, a gene required for dystroglycan processing, by Myf5-Cre induced a severe dystrophic phenotype, and a near-absence of functionally glycosylated dystroglycan (46). Lack of endogenous Myf5 expression in post-synaptic nuclei, fibroblasts, adipocytes, neurons, blood vessels and 10% of Pax7-positive satellite cells included in whole muscle may account for the apparent lack of recombination (46–48). Thus the total SMN detected in whole muscle is consistent with an 80% deletion rate indicated by the DNA recombination test.

Functional analyses of muscle force upon Smn-deletion

To investigate the integrity and function of muscle that was dependent on SMN from SMN2 (and SMN17), we performed physiological tests of twitch force (Fig. 5D), tetanic force (Fig. 5E) and eccentric-contraction-induced loss of force (Fig. 5F) on the extensor digitorum longus (EDL) muscle at 8 weeks postnatally. The investigators were blinded to the genotype of the mice. The muscle depleted for mouse Smn and dependent on SMN2 for full-length SMN showed no impairment of force production in any of the measures performed and was essentially identical to control muscle (n = 8 control males, n = 6 control females, n = 5 test males and n = 5 test females). Furthermore, Myf5-Cre; Smn<sup>D7/KO</sup> mice did not show any difference in muscle length (Fig. 5A), diameter (Fig. 5B) or weight (Fig. 5C), as compared with the controls, Myf5-Cre; Smn<sup>D7/WT</sup>. We also plotted the fiber size distribution of the vastus lateralis muscle at 8 weeks of age. The fiber size distributions of control (Myf5-Cre; Smn<sup>D7/WT</sup>, Fig. 5I) and test Myf5-Cre (Myf5-Cre; Smn<sup>D7/KO</sup>, Fig. 5J) mice do not differ (Mann–Whitney Rank Sum Test P = 0.926, n = 1350 fibers/group from three animals in each group). We also investigated the heart rate, although Myf5-Cre is not efficiently expressed in cardiac muscle. At 8 weeks of age, there was no significant difference in the unanesthetized heart rate, heart rate variability and QT interval (Supplementary Material, Fig. S3) of the control and Myf5-Cre test mice (n = 5 in each group). Thus we conclude that two copies of SMN2 (and SMNA7) provide sufficient full-length SMN for normal morphology and function of skeletal muscle.

Discussion

Proximal SMA exhibits a range of severity and has been classified into five main categories based on age of onset and motor milestones achieved (49). The most common form of SMA is Type I (Werdnig–Hoffmann disease), with the onset of symptoms before 6 months of age and patients not being able to sit (49). SMA Type II patients with onset by 18 months can sit, but do not walk (49). This is followed by the less severe form of Type III, Kugelberg-Weidner disease, wherein patients can walk, but have a waddling gait (49). The least severe form Type IV is adult onset with patients having the ability to stand and walk (49). A prenatal onset of SMA called Type 0 with severe asphyxia and early postnatal death has also been described (50). The copy number of SMN2 plays a major role in deciding the severity of SMA (10).

SMA is caused by insufficient levels of the SMN protein. The reduced levels of SMN do not occur only in motor neurons but also in other tissues such as muscle. A number of studies have indicated that muscle could play a role in SMA. Muscle extracts from SMA Type III patients have been shown to inhibit neonatal chicken neurite outgrowth, albeit with no cytotoxicity (51). We have previously reported that in severe SMA mice there is no abnormality of neurite outgrowth in vivo (52). Furthermore, muscle biopsy from all SMA Type I patients and most Type II, but not Type III, when co-cultured with normal rat motor neurons showed normal connection of the nerve to the muscle followed by sarcomeric disorganization and muscle degeneration (53). Guittier-Sigrist et al. showed apoptotic myonuclei and increased release of microparticles in SMA muscle co-cultured with rat motor neurons (54). The results would indicate an intrinsic defect in the muscle that only becomes apparent with connection to a nerve even though the nerve is normal. Further experiments revealed that when Type I SMA myoblast cells were mixed with 50% normal myoblasts cells, the hetero-myotubes corrected the degeneration phenotype seen in the co-cultures (55). The aforementioned results suggest that treatment of muscle could have a major influence on the SMA phenotype. However in the current paper we show that reduction of SMN to SMA-levels in muscle but not nerve does not result in any marked abnormality of muscle; in particular the contraction and force generating properties are normal. Studies in severe Smn<sup>−/−</sup>; Smn<sup>2/−</sup> SMA mice have shown that the number of satellite cells is comparable to wild-type controls (56). But when satellite cells of the severe SMA mice are cultured, they differentiate rapidly and fail to form multinucleated myotubes efficiently (56). In the mice in the current study, the development, function and structure of muscle is normal upon decreasing SMN in the muscle. Myf5-Cre will be expressed in myoblasts at embryonic day 8 and thus the aforementioned defects maybe present in myoblasts and satellite cells in the Myf5-Cre; Smn<sup>D7/KO</sup> mice reported here. However they do not lead to morphological or functional defects. This is also consistent with the recent report of Kariya et al. (57) which showed a marked reduction of SMN to levels produced by two copies of SMN2 at PND50 in all tissues including muscle. This SMN-reduction did not result in a dramatic phenotype and grip strength was not reduced at 120 days. Some pathology of muscle did occur at 230 days with increase in central nuclei, which indicates muscle regeneration from satellite cells. It can be noted that central nuclei is not a prominent feature of SMA muscle biopsies particularly in the milder forms of the disease (58). One factor that we have not examined is the ability of the SMN-deficient muscle to...
perform repair. Indeed, MyoD−/− mice with a homozygous deletion of an early myogenic regulatory factor, MyoD, appear phenotypically normal. MyoD−/− mice do not show a marked phenotype of deficiency in repair unless they are put under stress; in the case here by crossing with mdx mice which lack dystrophin (59).

SMA mice and SMA muscle have been examined previously in the situation where SMN is deficient everywhere. Cell culture studies of SMA muscle have shown abnormalities in myoblast fusion and malformed myotubes along with delayed expression of myogenic proteins (60–62). The results have been supported by in vivo studies showing defects in myogenic program (61) and apoptosis in muscle (63) in SMA mouse models. Furthermore, skeletal muscle from SMA Type I fetuses exhibit decreased myofiber diameter and fiber size distribution (64), and abnormalities in early markers of muscle development (65), leading to the hypothesis of delayed growth and maturation of skeletal muscle in SMA. A total of 54% of SMA Type I, II and III cases sampled in a study showed DNA fragmentation and immaturity of muscle fibers (66). In the studies mentioned above, muscle appears to show a lack of maturity suggesting a delay in myogenesis. In addition, muscle in this state shows a reduced ability to produce force (67). However there are two difficulties in interpreting

Figure 5. Muscle physiology tests on mice with SMN-reduction in the muscle, Myf5-Cre; SmnD7/KO, at 8 weeks: There was no significant difference in the (A) Length, (B) Diameter, (C) Weight, (D) Twitch force, (E) Maximal Tetanic force and (F) Eccentric-contraction-induced loss of force of the EDL muscle of control (Myf5-Cre; SmnWT) and affected (Myf5-Cre; SmnD7/KO) mice (Control: n = 8 males and n = 6 females, Test: n = 5 males and n = 5 females). Representative H&E stained Vastus lateralis muscle of (G) control and (H) affected mice at 8 weeks of age and the muscle fiber size distribution of (I) control and (J) affected mice show no significant difference in morphology (a total of n = 1350 fibers/group from three animals in each group, Mann–Whitney Rank Sum Test, P = 0.926, Error bars = sem).
these results: first, under conditions of denervation, the muscle atrophies. The fibers are smaller and likely not as developed therefore can produce less force. Indeed atrophied muscle due to disuse shows a number of changes and reduced capacity to produce force (68). This effect of decreased force production is likely independent of SMN’s requirement in muscle. Second, no experiments were done to restore SMN in the muscle in vivo; for instance by using AAV-SMN to determine whether these alterations were reversible. Lastly Cifuentes-Diaz et al. (24) reported that complete removal of Smn from muscle resulted in a dystrophic phenotype. However in SMA, the SMN2 gene produces some full-length SMN and the critical question is whether this low amount of SMN is sufficient for normal function of muscle. Also, we have not observed loss of dystrophin staining in SMNΔ7 SMA mice (32).

In the current paper we found no apparent change in the phenotype of mice upon SMN-reduction in muscle, with the total body weight, fiber size distribution and survival comparable to healthy controls. Furthermore, at 8 weeks there was no change in twitch and tetanic force or eccentric-contraction-induced loss of force of EDL upon Smn-deletion in the muscle. The mice with Smn-replacement in the muscle were phenotypically similar to SMA mice, having a median survival of 16 days as compared with 14 days for SMA mice. Martinez et al. (37) reported a median survival of 21 days in Myf5-Cre; Smn-replacement mice. An increase in muscle area and myofiber diameter was also reported in the Smn-replacement mice in this study (37). The slightly increased survival obtained in this study most likely relates to the SMA mice having two of the replacement alleles, i.e. Myf5-Cre; SmnRex1Rex1. The SMA mice without Cre, SmnINV/INV, had a median survival of 15 days (37). We have also previously reported that extremely high levels of SMN in muscle specifically, with no expression in other tissues, did not improve survival, weight or fiber size (23). Both in the current study and previous study we looked at the complete distribution of muscle fibers and found no difference with SMN overexpression in muscle. An HSA-SMN line with leaky expression in the nervous system significantly increased the mean survival of SMA mice to 160 days (23). Complete deletion of SMN in the muscle using the HSA-Cre driver resulted in severe muscle dystrophy and a drop in survival to a mean of 33 days but loss of SMN in all tissues tested to date results in severe problems (24). One important consideration with the use of HSA-Cre is whether there is any significant ectopic expression of the driver, as this has not been fully tested for HSA-Cre driver and we have previously shown that HSA can, in some transgenes, have ectopic expression (23). In the latter case weak but clear expression could be observed throughout the nervous system (23). The specificity and strength of the Cre driver is an important consideration. Here we showed strong tdTomato-REF expression in all muscle tissue using a loxP-STOP-loxP-tdTomato line as well as efficient deletion of SMN exon7 in muscle using laser microdissection and ddPCR. There is occasional and rare expression of Myf5-Cre in MNs but this is in only certain animals and only a few motor neurons in a section; so unlikely to have a major impact as the remaining motor neurons will sprout and compensate for the loss of only a few motor neurons. Expression in a patch of cells was observed for this Myf5-Cre line in the heart, lung, liver, pancreas and kidney in some, but not all animals examined. It is unlikely for a patch of Cre expressing cells in a tissue to significantly contribute to the overall phenotype. Another important aspect of muscle is that it is a syncytia with essentially a tube containing multiple nuclei. Given the volume of a muscle fiber [5 nl for a mouse limb fiber and five orders of magnitude larger that of a small uninnucleated cell (69)], the presence of one nucleus at the post-synaptic plate is unlikely to make major contribution over the whole muscle. It is also well known that proteins in muscle can have a relatively restricted nuclear domain with the product produced by that nucleus restricted to a relatively tight region (68–74). In the current experiments we have shown that at least 80% of the muscle nuclei have loss of the mouse Smn exon7 and thus become reliant on SMN2. This also resulted in a drop of SMN protein in muscle. Given the large volume of a muscle cell and the nuclear domain, this should result in a myopathic phenotype being revealed and an abnormal force of contraction being present. However this is not the case and thus we conclude that two copies of SMN2 provide sufficient SMN for normal function of muscle.

We are of the opinion that it is important to perform both replacement and removal of SMN from a tissue to fully understand its role. Thus in the current paper it is clear that reduction of SMN to SMA-levels does not have a major impact on muscle and indicates a minimal role for muscle in SMA.

Many studies have targeted muscle for therapy and amelioration of atrophy. Follistatin injections which block myostatin thus enhancing muscle mass, resulted in weight increase and a mild increase in survival (28). Transgenic overexpression of follistatin or inactivation of myostatin did not ameliorate the disease in SMA mice (27,29). We have previously shown that deletion of muscle ubiquitin ligases, MARbx and MuRF1, which target the muscle proteins for degradation, did not alter the disease course in SMA mice (30). Treatment of SMNΔ7– SMA mice with Fasudil, an inhibitor of RhoA/Rho kinase pathway, improved the muscle fiber size but did not improve the performance (75). Thus even therapies that target various aspects of muscle have so far had minimal impact.

Various therapeutic strategies targeted towards the central nervous system have been explored in SMA. Gene therapy with delivery of SMN in an scAAV9 viral vector increased the survival of SMA mice beyond 250 days (76–78). Second, anti-sense oligonucleotides that trick the SMN2 gene into splicing like SMN1 and thus increase the production of full-length SMN have been successful in increasing the life span and improving the phenotype in SMA mice (79–81). Third, from a wide-array of compounds, specific compounds that modulate the splicing of SMN2 gene and promote the incorporation of exon 7 were screened for (82). Daily oral dosing of three promising compounds in SMA mice showed a marked increase of full-length SMN in brain, spinal cord and muscle along with an increase in survival and body weight (82). Increased SMN expression in the neurons is an important factor for improvement of the SMA phenotype in the above-mentioned therapies. Recently, the importance of high SMN in the peripheral tissues has been indicated (83). We have also examined neuronal SMN and we discuss the role of the neurons and the periphery in the accompanying manuscript (84). Given our results in the present work where deletion of Smn in the muscle did not decrease the muscle fiber size or contractile function, and replacing Smn solely in the muscle did not improve the survival or weight of SMA mice, we conclude that low SMN levels are sufficient for normal muscle function.

Materials and Methods

Mouse breeding

The Cre lines and the floxed Smn lines were crossed to the SMNΔ7 SMA mouse model (SmnWT/KO, SMN2Δ7; SMNΔ7Δ7—Jackson No. #005025, FVB.Cg-Tg(SMN2)89Ahmb Smn1tm1(Smad-Tg(SMN2*delta7))
Genotyping, weighing and phenotypic assessment of mice

Genomic DNA was isolated from tail clips for genotyping. The primers used for PCR are described in Table 3. The PCR conditions and genotyping of SmnKO, Smn2 and SmnA7 were as described in Le et al., 2005 (32). The mice were weighed daily from day of birth, PND0 to weaning, to weaning at PND21, and weekly thereafter. Phenotype was observed every day till weaning, and then weekly. Mice were sacrificed according to our IACUC approved protocol. Approximately equal number of males and females were studied in each genotypic grouping. The number of animals required for analysis was determined as described previously (85).

Histology and immunohistochemistry

Hematoxylin and Eosin (H&E) staining was performed on 14 µM muscle sections as described (32) and imaged on Nikon Eclipse 800 microscope (Nikon Corporation, Japan) with Nikon FDX-35 digital camera. Morphometric measurements were done using SPOT Advanced (v3.5.9) software (Diagnostic Instruments, Inc., MI, USA), after binning the frequency of fiber size area on Microsoft Excel 2007.

For Immunohistochemical staining, PND12 mouse pups were perfused with PBS followed by 4% paraformaldehyde (pH 7). The tissues post-harvesting were infused with 30% sucrose and flash frozen in isopentane cooled to −150°C in liquid nitrogen. The frozen tissue was embedded in Tissue-Tek OCT (Fisher Scientific) and cryostat (IEC Minotome Plus) sectioned. A total of 14 µM thin sections were blocked with goat serum (30 min), and stained with Chicken anti-red RFP (1:2500, Millipore AB3528) and Rabbit anti-GFP (1:1000, Molecular Probes A11122) for 2 h. Secondary antibody incubation (Alexa-488, Alexa-594 at 1:1000 each) was done before and after application of secondary antibody. Fluoromount-G (Southern Biotech) was used for mounting the tissues. SMN was similarly stained with mouse anti-SMN (1:500, BD Biosciences 610647; Alexa-488 secondary) and DAPI.
was used to visualize the nucleus. All confocal imaging was performed using Leica DM IREZ with Leica TCS SL point scanning laser confocal system with photomultiplier tube detection. Subsequent image processing was done using Adobe Photoshop CS2.

**Muscle physiology tests**

In both male and female mice, at age of 8 weeks, muscle function test were performed, with the investigator being blinded to the genotype. First, electrocardiograms were obtained in unrestrained, anaesthetized mice using the ECGenie system. Briefly, the mice were allowed to sit on a platform that registered ECG-signals through a set of three electrodes located on the platform. From the obtained recordings, heart rate, heart rate variability and QT interval, an important indicator for cardiac muscle dysfunction, were assessed. Next, after cervical dislocation, the EDL muscle was dissected, and mounted into a muscle physiology set-up, and functional assessment of contractions were assessed as previously described (86). Briefly, after stretching the muscle to optimal length, using twitch-contraction force as a read-out, a maximal tetanic force was achieved by applying a 250 ms duration 180 Hz stimulation train (1 ms per pulse) to the EDL muscle. Thereafter, a series of 10 eccentric contractions were performed, in which the muscle was stimulated for 700 ms total duration (180 Hz pulses of 1 ms duration), while in the last 200 ms the muscle length was linearly increased by a servo-controlled motor, for a total stretch of 3% of the muscle length. At \( t = 700 \) ms, the stimulation was halted and the muscle was returned within 200 ms to its original optimal length. The muscle was allowed to rest for 2 min in between contractions. After assessment of contractions, the muscle was removed from the set-up, blotted dry in between 2 kimwipes using a 10 g weight for 10 s and muscle weight was assessed. All obtained forces were normalized to the cross-sectional area of the muscle to calculate specific force of the muscle.

**H&E staining for LCM**

Slides for LCM (Zeiss Membrane Slide 1.0 PEN, 415190-9041-000) were treated with UV radiation for 30 min just before blotting 14 µm gastrocnemius sections on to them. For H&E staining, the slides were fixed in 70% ethanol (1 min), followed by 50% ethanol (1 min) and H2O (3x over 1 min). Muscle sections were stained with Weigert’s iron Hematoxylin (Sigma) for 1 h, rinsed in dH2O, destained in 0.3% acid alcohol (5–7 dips) and rinsed in tap water. Next, the sections were stained with Eosin for 5 s (Sigma) and dehydrated with 70, 90 and 100% ethanol, air-dried shortly (10 min) and stored at 4°C.

**Laser-capture microdissection (LCM)**

Laser-capture microdissection was performed with the Palm Microbeam (Carl Zeiss MicroImaging) under 10× magnification. Approximately 150 000–200 000 µm² of muscle tissue was collected from H&E stained slides, avoiding the extra-sarcomeric tissue. The tissue was collected in 18 µl of cell-lysis solution containing 20 mM Tris–HCl pH 8, 0.02% Tween and 1 mM EDTA and frozen on dry ice. Before performing ddPCR, 2 µl of 2 mg/ml proteinase K (freshly prepared 1:10 dilution of 20 mg/ml of proteinase K (Invitrogen) in a solution of 20 mM Tris–HCl pH 8, 0.1 mM EDTA) was added to the sample. The sample was heated at 55°C for 2 h, followed by inactivation of proteinase K at 90°C for 10 min. Silicogenized tubes (87) and low-bind tips (Eppendorf) were used throughout the experiment to prevent the loss of low amounts of DNA via adsorption onto plastic.

**Droplet digital PCR (ddPCR)**

LCM-collected tissue was divided into two parts and multiplex ddPCR was thus performed in duplicates with each sample. After addition of primers, probes and ddPCR SuperMix (BioRad), the sample was partitioned into approximately ten to sixteen thousand droplets and PCR was performed (79). The fluorescence signal was measured and calculated according to Poisson statistics (79) and analyzed using QuantaSoft (BioRad) software. The sequence of the primers and probes are as follows: Smn exon 7—FP 5′-AAGATGCTTGGAACTCGGGTCTC, RP 5′-TGTGA GTGAACATTCAAGCCC, Probe—5′-FAM-CTGGCATGCACTACC AAAGCTTG-CAT-MGB; Smn intron 1—FP 5′-CTGTGTAACGTGTTGA GGGATGTCG, RP 5′-CCTGTAACATCTTCTCTCCTGACCTAA, Probe—5′-VIC-AGGCTTGCTGAAGCAAGG CAACCAGATA-MGB. Eppendorf LoBind tips were used throughout to prevent loss of DNA.

**ELISA on whole muscle sections**

Gastrocnemius muscle of PND10 mice was flash frozen in isopentane cooled to −150°C in liquid nitrogen and cryostat (IEC Minotome Plus) sectioned. 2–4 sections of 14 µm thickness were collected in 100 µl of ELISA-lysis solution (PharmaOptima, Portage, MI, USA). A total of 40 µl of whole muscle section lysates were diluted 1:2 with sample buffer to a final volume of 80 µl. 25 µl of the lysate was used in the SMN ECL immunosassay. The assay is a quantitative sandwich immunosassay, where a mouse monoclonal antibody (281, Liu and Dreyfuss 1996) functions as the capture antibody and a rabbit polyclonal anti-SMN antibody (ProteinTech, Cat. No. 11708-1-AP) labeled with a SULFO-TAG™ is used for detection. SMN levels are determined from a standard curve using recombinant SMN protein (Enzo Life Sciences, Cat. No. AD1-NBP-201-050) as calibrator. The dynamic range of the assay is 10 pg/ml (lower limit of quantitation) to 20 000 pg/ml (upper limit of quantitation). Assay plates were read using a Meso Scale 6000 sector imager. 5 µl of non-diluted lysate was used for Pierce BCA protein assay. SMN levels were then normalized to total soluble protein.

**Western blot analysis**

Western blots were performed as previously described (23,79,88). Briefly, 50 µg of protein (skeletal muscle, PND10 pups) was loaded per lane and SMN was detected with mouse anti-SMN (1:1500, BD Biosciences, 610647) while tubulin was detected with mouse anti-Tubulin (1:25 000, Sigma, T8203). Mouse-anti HRP (1:10 000, Jackson Immuno Research) was used as the secondary antibody and the blot was visualized using the ECL system, as described by the manufacturer (GE Healthcare Life Sciences). Blots were scanned and quantified as described (http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/) and the area under each peak determined with ImageJ software.

**Statistical analyses**

Survival (Kaplan–Meier curve) and statistical analyses (Mann–Whitney Rank Sum Test,Shapiro–Wilk Normality Test) were done using SigmaPlot v12.0 (Systat Software Inc., CA, USA). Weight curves were analysed using Statmod (89,90). t-tests were performed using Microsoft Excel 2007.
Supplementary Material

Supplementary Material is available at HMG online.

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