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Review

Pig models for Duchenne muscular dystrophy – from disease mechanisms to validation of new diagnostic and therapeutic concepts

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Abstract

Duchenne muscular dystrophy (DMD) is a fatal X-linked disease caused by mutations in the *DMD* gene, leading to complete absence of dystrophin and progressive degeneration of skeletal muscles and heart. Animal models are essential for preclinical evaluation of novel diagnostic procedures and treatment strategies. Gene targeting/editing offers the possibility of developing tailored pig models for monogenic diseases. The first porcine DMD model was generated by deletion of *DMD* exon 52 (*DMD*Δ52) in cultured kidney cells, which were used for somatic cell nuclear transfer to produce *DMD*Δ52 offspring. The animals resembled clinical, biochemical, and pathological hallmarks of DMD, but died before sexual maturity, thus preventing their propagation by breeding. This limitation was overcome by the generation of female heterozygous *DMD*Δ52 carrier pigs, which allowed the establishment of a large breeding colony. In this overview, we summarize how porcine DMD models have been used for dissecting disease mechanisms, for validating multispectral optoacoustic tomography as an imaging modality for monitoring fibrosis, and for preclinical testing of a CRISPR/Cas9 based approach to restore an intact *DMD* reading frame. Particular advantages of porcine DMD models include their targeted design and the rapid disease progression with early cardiac involvement, facilitating translational studies in reasonable time frames.

Keywords: Duchenne muscular dystrophy; Pig model; Optoacoustic imaging; Gene editing.

1. Introduction

Duchenne muscular dystrophy is a fatal, X-chromosomal, neuromuscular disorder caused by mutations disrupting the

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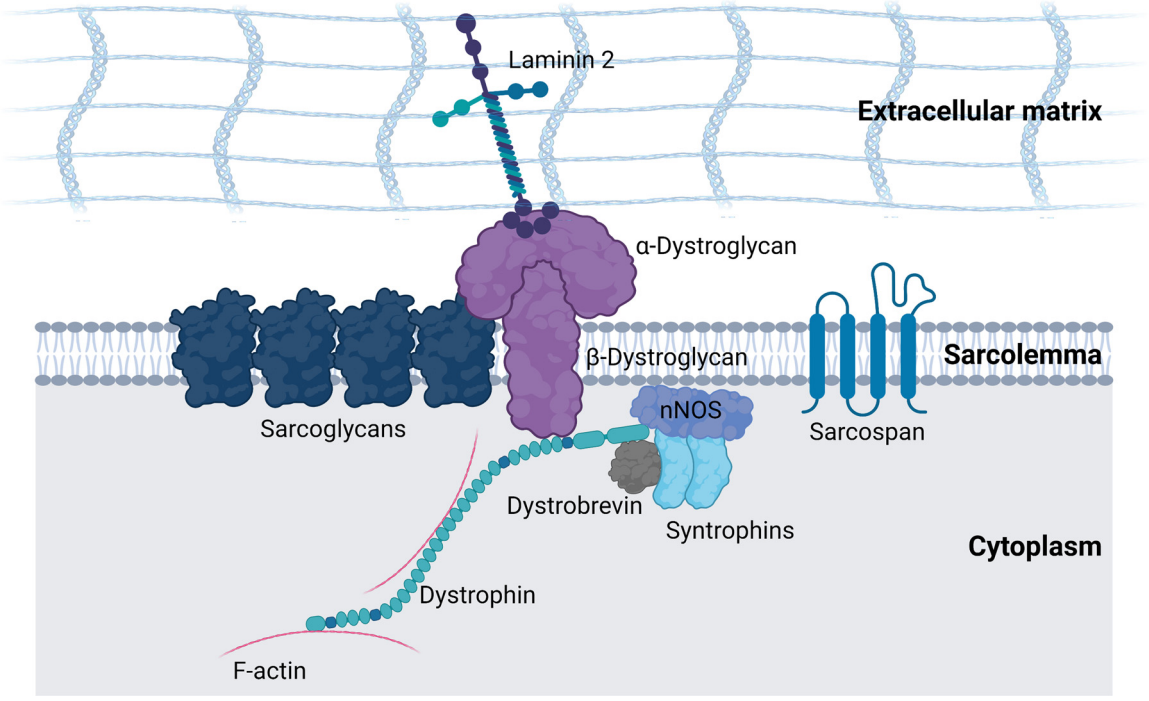

Fig. 1. Simplified drawing of the dystrophin-associated protein complex (DAPC). Dystrophin connects the cytoskeleton of (cardio-)myocytes, via several glycoproteins, to the surrounding extracellular matrix. Intracellular, the F-actin filaments of the cytoskeleton bind to the N-terminal region and the 11.−15. 'spectrin-like' repeats of the dystophin protein ('spectrin-like' repeats are shown as turquoise ovals), while the C-terminus links the dystrophin to β-dystroglycan. The β-dystroglycan in turn provides the link between the dystrophin and the extracellular matrix, via α-dystroglycan and laminin 2. The following additional glycoproteins modulate the binding of the proteins already mentioned: α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan, δ-sarcoglycan, α-syntrophin, β-syntrophin, nNOS, dystrobrevin. Created with BioRender.com.

reading frame of the 2.2-Mbp dystrophin gene [*DMD, ensembl reference ENSG00000198947 (H. sapiens) and ENSSSCG00000028148 (S. scrofa)*]. Most common are intragenic deletions of one or more exons, with a major mutation hotspot region spanning exons 45 to 53 (reviewed in [1]). Mutations in the *DMD* gene occur more frequently than expected from the average mutation rate, resulting in one affected boy in about 5000–6000 male births (reviewed in [2]). *DMD* frame-shift mutations cause premature stop codons, leading to prematurely truncated, dysfunctional proteins or nonsense-mediated mRNA decay, and the complete absence of functional dystrophin, an essential protein for skeletal and cardiac myocytes. Dystrophin connects the actin cytoskeleton via a large complex of dystrophin-associated glycoproteins with the extracellular matrix (Fig. 1). The loss of dystrophin results in muscle fiber damage, manifesting in progressive muscle weakness, loss of ambulation, dependence on artificial respiration, and early death due to cardiac or respiratory failure (reviewed in [3]). To date, no cure exists and glucocorticoids are still the state-of-the-art treatment in DMD patients, even though they can only slow down disease progression (reviewed in [4]). A variety of anti-inflammatory and novel pharmacological drugs are being tested in clinical trials, such as vamorolone (a steroid with corticosteroid anti-inflammatory activity but fewer side effects), NF-κB-, myostatin-, and histone deacetylase inhibitors as well as anti-connective tissue growth factor (CTGF)/cellular communication network factor 2 (CCN2) antibodies. In addition, a variety of therapies is aiming at restoration of dystrophin, including drugs providing stop codon read-through (such as ataluren), antisense oligonucleotides for exon skipping (such as eteplirsen), and adeno-associated viral vectors (AAV) for microdystrophin expression or for the delivery of Cas9/guide RNAs to delete specific *DMD* exons for restoration of an intact reading frame (reviewed in [5]).

Animal models have been essential for the development of these new therapeutic concepts. During the last decades, two animal models were mainly used for DMD research: the *mdx* mouse with a nonsense point mutation in exon 23 [6] and the Golden Retriever Muscular Dystrophy (GRMD) dog with a splice site point mutation at intron 6, leading to splicing of exon 7 during RNA processing and a shift in the reading frame (reviewed in [7]). None of these animal models resemble the most common *DMD* mutations in humans. Therefore, several other *mdx* mouse strains with patient-relevant mutations, including *Dmd*Δ52 [8], have been generated by gene targeting (reviewed in [9]), but dystrophin deficient mice show only moderate histopathology and a slightly reduced life span. Additional inactivation of the utrophin (*Utrn*), α-dystrobrevin (*Dtna*), α7-integrin (*Itga7*), myogenic differentiation 1 (*Myod1*), putative cytidine monophosphate-N-acetylneuraminic acid hydroxylase-like protein (*Cmah*), or the telomerase RNA

component (*Terc*) genes worsens the phenotype of *mdx* mouse models (reviewed in [9]). This spectrum of mouse models for DMD research has had a tremendous impact in terms of understanding disease mechanisms and testing new therapeutic approaches. However, for translational studies large animal models are additionally required. In addition to the original GRMD dog model [10] and beagle-based canine X-linked muscular dystrophy in Japan ($CXMD_J$) [11], several other lines with canine X-linked muscular dystrophy have been selected (reviewed in [7,12]). Among them is Cavalier King Charles Spaniels with a missense mutation in the 5′ splice site of *DMD* exon 50 that results in deletion of exon 50 in mRNA transcripts and a predicted premature truncation of the translated protein. In the Cavalier King Charles Spaniel muscular dystrophy (CKCS-MD), an intact *DMD* reading frame can be restored by skipping exon 51 [13]. The model is thus suitable for evaluating a therapeutic approach that benefits a large proportion of DMD patients. Although canine DMD models have been successfully used for preclinical studies ([14–22]; (reviewed in [23,24]), their use as experimental animals is associated with ethical concerns and difficulties in breeding and maintenance [25]. Moreover, GRMD dogs show clinical signs of cardiomyopathy not earlier than age 2.5 years [26]. In $CXMD_J$ dogs, dystrophic changes of myocardium were not evident up to an age of 13 months [27], but vacuolar degeneration of cardiac Purkinje fibers was observed already at age 4 months [21,28]. In addition, DMD models have been created in several other species, including rabbit [29], rat [30,31], and rhesus monkey [32].

Over the last two decades, the pig has developed to an interesting medical model organism for many disease areas (reviewed in [33]). Major advantages of this species include i) many similarities with human anatomy and physiology; ii) the possibility of efficient and precise genetic modifications; iii) high fecundity and fast maturation; iv) established maintenance systems under specified pathogen-free (SPF) conditions; and v) less ethical restrictions of their use compared to nonhuman primates or dogs. Recent resources, such as standardized tissue sampling protocols [34] and a pig BodyMap transcriptome database [35] further raise the value of the pig as a biomedical model species. The rapid growth rate of pigs makes them a particularly attractive model species for genetic muscle diseases, since clinically severe disease phenotypes are expected in an accelerated mode (reviewed in [36]).

The rapid disease progression may explain why – in contrast to dogs – no spontaneous pig model for DMD has been reported. There are, however, reports on milder forms of muscular dystrophy. Nonneman et al. [37] identified in pigs showing an increased stress response an arginine to tryptophan (R1958W) polymorphism in exon 41 of the *DMD* gene, which was associated with reduced abundance of dystrophin, compromised assembly of the dystrophin glycoprotein complex, increased serum creatine kinase activity, and signs of muscular dystrophy. These pigs were thus considered as a novel model for Becker muscular dystrophy (BMD) ([38]; reviewed in [39]). Additional cases of "BMD-like myopathy" [40] or "fatty muscular dystrophy" [41] were reported without identifying the underlying mutations.

Here, we review different strategies for the development of porcine DMD models, their clinical phenotypes and pathological characteristics including molecular alterations, and their use for preclinical testing of new diagnostic tools and therapeutic concepts.

2. Development of pig models for Duchenne muscular dystrophy

The first porcine DMD model was generated by deletion of *DMD* exon 52 in male porcine kidney cells (Fig. 2A) that were subsequently used for somatic cell nuclear transfer (SCNT) to generate *DMD*Δ52 pigs (Fig. 2B) [42]. Deletion of *DMD* exon 52 was achieved by homologous recombination with a modified bacterial artificial chromosome (BAC) vector in which exon 52 was replaced by a neomycin resistance (*neo*) cassette. The cloned offspring ($n = 24$ in total) showed a marked variation in birth weight, which appeared to be inversely correlated with life expectancy. The majority of the piglets died within the first week of life, and none of them survived longer than 3.5 months. The severe progressive muscular dystrophy (see below) prevented propagation of the model by breeding.

An elegant strategy to overcome the problem of propagating pig models with severe X-linked diseases was proposed by Matsunari and coworkers [43]. The concept is based on complementation of male embryos carrying the mutation with blastomeres from female wild-type (WT) embryos. In the resulting chimeric offspring, the female cells – depending on the extent of chimerism –partially rescue the respective phenotypic lesions caused by the mutation of the male cells. However, since the male cells suppress female sexual development, the phenotypically male chimeras produce sperm transmitting the mutation to the next generation (Fig. 2C). In case of the *DMD*Δ52 mutation [42], two chimeric boars were born. One with a low proportion of female cells showed the characteristic DMD phenotype and died early at an age of 3 months. The other boar revealed a higher proportion of female cells and reached sexual maturity. Epididymal sperm could be collected at an age of 7 months and is available for producing *DMD* mutant pigs by in vitro fertilization. However, this animal showed mild symptoms of muscular dystrophy, like forced breathing and reduced muscle strength. Histologically, the two chimeric boars revealed different proportions of mutated/WT cells, which explains the different severity of the DMD symptoms.

Another possibility is the generation of female carrier animals with the *DMD*Δ52 mutation (Fig. 2D). Using the BAC targeting strategy of *DMD* exon 52 deletion developed by Klymiuk, Blutke [42] in female pig cells, several $DMD^{+/-}$ carriers could be established by SCNT. One of them (#3040) reached sexual maturity and gave birth to 11 litters with a total of 29 $DMD^{Y/-}$, 34 $DMD^{+/-}$, as well as 65 male/female

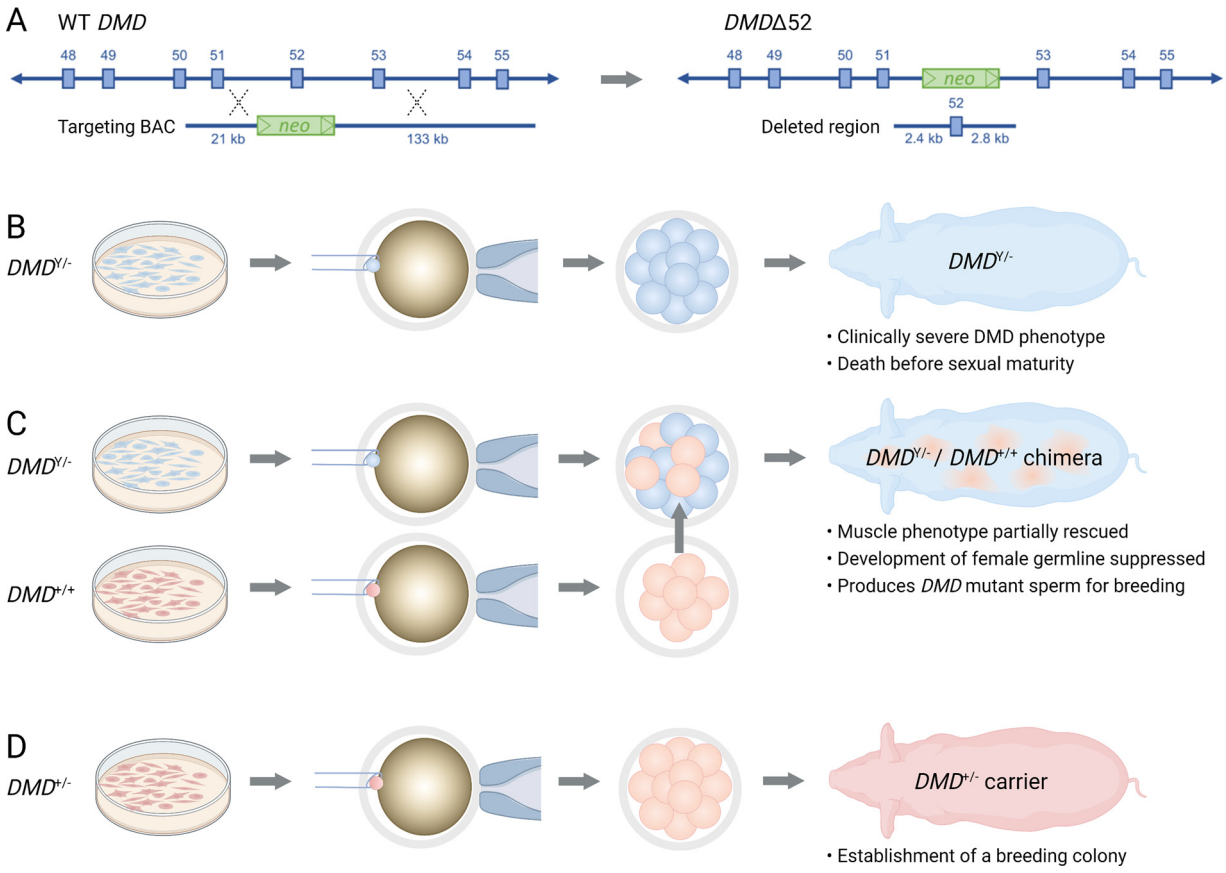

Fig. 2. Different strategies towards the development of DMD pig models. (A) Exon 52 of the *DMD* gene was replaced by a neomycin resistance cassette (neo), by using a modified bacterial artificial chromosome (BAC) in a male porcine kidney cell line. (B) *DMD* exon 52 deficient (*DMD*Δ52) cells were used for somatic cell nuclear transfer (SCNT) and the resulting cloned embryos transferred into the oviducts of estrus-synchronized recipients. (C) Generation of chimeric boars, through complementation of male *DMD*Δ52 SCNT embryos with blastomeres from female wild-type embryos. (D) BAC targeting of a female cell line, resulted in a heterozygous ($DMD^{+/-}$) carrier sow, which was used to establish a breeding herd. Created with BioRender.com.

WT offspring. Breeding with F1 and F2 $DMD^{+/-}$ carriers resulted in additional 114 $DMD^{Y/-}$ piglets. With intensive neonatal care, the majority survived for 3–4 months, providing statistically relevant cohort sizes for experimental studies [44].

Other *DMD*Δ52 pigs were produced by rAAV-mediated gene targeting in male Yucatan miniature pig cells and subsequent SCNT [45]. In total, 7 piglets were born. Five of them died within the first week after birth, while the other two became 6.5 and 7 months old.

In addition to gene targeting, gene editing (CRISPR/Cas9) was used to establish pigs with *DMD* mutations. In the first study, Diannan miniature pig zygotes were injected with Cas9 mRNA and single-guide RNA specific for *DMD* exon 27. After transfer of 98 injected embryos to 8 estrus-synchronized recipients, two piglets were born. One of them turned out to be unaffected, while the other showed mosaicism of different proportions in different organs (20–80%) with multiple in-frame and frameshift mutations of exon 27 [46]. A more recent study used CRISPR/Cas9 to mutate *DMD* exon 51 in a male Bama miniature pig cell line. From 200 SCNT embryos transferred to 5 synchronized recipients, 15 piglets were born of which only 9 carried the *DMD* exon 51 mutations (suggesting the use of mixed cell colonies). All *DMD* mutant pigs died within the first 12 weeks of life [47].

3. Clinical and pathological characteristics of DMD pigs

Characterization of the first cohort of DMD pigs produced by SCNT [42] showed reduced mobility and markedly elevated serum creatine kinase (CK) levels as a sign of muscle damage already present in newborn animals. The 24 cloned DMD pigs analyzed by Klymiuk, Blutke [42] showed a large variation in body weight, which is a possible side effect of the SCNT technology [48]. Interestingly, there was a negative correlation between birth weight and life expectancy. Animals with a birth weight of more than 1200 g died within the first few days with clinical symptoms suggesting muscle weakness and breathing problems as primary causes of death. In contrast, the life expectancy of animals with a relatively low birth weight (< 700 g) was in the range of 3 months. This observation points to an effect of intrauterine and postnatal growth rate on the severity of the disease. In particular, rapid muscle growth of pigs by hypertrophy of muscle fibers and the associated increased mechanical strain on the sarcolemma is a possible explanation for the faster progression of the

muscular dystrophy compared with DMD in human patients. Interestingly, dystrophin-deficient Yucatan miniature pigs with a smaller growth rate compared to farmed pigs survived for up to 7 months [45].

A detailed cinematographic analysis of a cloned DMD piglet (age 9 weeks) showed disturbed mobility in all three gaits (walk, trot, gallop), with shortened strides and stiff movements being the most prominent features. A test involving climbing to a small platform demonstrated striking muscle weakness. Muscle weakness was seen in a chimeric DMD pig with low contribution of healthy female cells, whereas a high contribution of healthy cells rescued this phenotype [43]. Since respiratory and laryngeal muscles are affected, the animals often showed forced breathing at a later stage [42,43].

The clinical observations in cloned DMD pigs [42] were largely recapitulated in $DMD^{Y/-}$ pigs generated by breeding of $DMD^{+/-}$ carrier pigs [44]. An exception was body weight, which showed less variation but was consistently lower in $DMD^{Y/-}$ than in WT littermates. Nevertheless, a large proportion (42%) of the $DMD^{Y/-}$ piglets died within the first week of life. With intensive neonatal care, this proportion could be reduced to 23%, and the majority of the animals survived for 3–4 months. Only a few ($n = 3/143$) $DMD^{Y/-}$ pigs survived for more than 6 months (maximum 9 months).

Serum creatine kinase activities in $DMD^{Y/-}$ pigs and in part also in $DMD^{+/-}$ carrier females were highly elevated. $DMD^{Y/-}$ pigs showed a reduced activity profile [49] and – in line with observations in DMD patients phenotype [50] and *mdx* mice [51] – signs of cognitive impairment as revealed by two different behavioral tests [44]. Specifically, in a novel object recognition test measuring the times spent for exploring a known and a new object, the total exploration time for both objects was significantly shorter in $DMD^{Y/-}$ than in WT pigs. Moreover, $DMD^{Y/-}$ pigs revealed a significantly increased failure rate in the first test round of a black and white discrimination test.

Importantly, echocardiography revealed impaired systolic cardiac function as indicated by a significantly reduced left ventricular ejection fraction (-19%) and left ventricular fractional shortening (−27%) already in 4-month-old $DMD^{Y/-}$ pigs. Thus, early stages of dilated cardiomyopathy as observed in adolescent DMD patients (reviewed in [52]) occur much earlier in the porcine DMD model than in GRMD dogs (>30 months; [26]).

It is worth mentioning that several $DMD^{Y/-}$ pigs (11 of 28 animals investigated) showed gastrointestinal symptoms, such as difficulties in defecation (repeated strain to eliminate feces), increasing abdominal girth, anorexia, or abdominal edema and ascites (detected by ultrasound scan) within the first weeks of life. The model thus recapitulates gastrointestinal dysfunction including constipation present in patients with a later stage DMD [53].

Systematic pathological analysis of cloned DMD pigs [42] revealed pale skeletal muscles of moist texture, with multifocal areas of pale discoloration, especially in the diaphragm and intercostal musculature. Histological examination revealed a polyphasic myopathy with excessive fiber size variation, numerous large rounded hypertrophic fibers, branching fibers and fibers with central nuclei, as well as scattered clusters of segmentally necrotic fibers, next to hypercontracted fibers and groups of small regenerating muscle fibers. These lesions were accompanied by interstitial fibrosis and mononuclear inflammatory cell infiltration, mimicking the hallmarks of the human disease. The severity and extent of these alterations progressed with age (Fig. 3A) and was most severe in the diaphragm, the laryngeal and intercostal musculature, and the triceps brachii muscle. Examination of the myocardium of cloned DMD pigs < 3 months of age did not reveal accentuated signs for a cardiac involvement.

Morphometric analyses of biceps femoris muscle samples revealed that the mean minimal Feret's diameter of muscle fibers was 34% ($p<0.01$) and 55% ($p<0.001$) reduced in 2-day-old and 3-month-old DMD pigs as compared to age-matched WT controls (Fig. 3B). While the distribution of muscle fiber diameters from the mean was similar in 2-day-old DMD and WT pigs, 3-month-old DMD pigs displayed a broadened, biphasic distribution, with peaks at small and large fiber diameters, indicating progressive DMD pathology [42]. Furthermore, the proportion of muscle fiber cross section profiles with centrally located nuclear section profiles was doubled in 2-day-old DMD pigs and was increased by more than 20-fold in 3-month-old DMD pigs (Fig. 3C).

These pathological changes were largely recapitulated in $DMD^{Y/-}$ pigs produced by breeding [44]. In the few $DMD^{Y/-}$ pigs in this cohort that survived for more than 6 months, also histological changes of the myocardium were observed, in particular degenerating and necrotic cardiomyocytes with interstitial edema, and infiltration of macrophages, accompanied by considerable increased numbers of eosinophils.

Necropsy of animals euthanized after showing gastrointestinal symptoms revealed accumulation of gas and ingesta in the jejunum and large intestine, associated with obstipation, dark coloration of the intestinal wall, infarction of the intestinal wall and/or ascites and fibrinous layers in the abdominal cavity. Histomorphological evaluation revealed increased collagen deposition (a sign of fibrosis) in several parts of the gastrointestinal tract [44]. Corresponding gastrointestinal alterations were also observed in cloned Bama minipigs with *DMD* exon 51 mutations [47].

A recent quantitative stereological study of somatotroph cells in the anterior pituitary gland did not provide evidence for a pituitary dysfunction to be a cause for the reduced growth of DMD pigs [54].

In summary, clinical and pathological studies indicate that DMD pigs develop a progressive muscular dystrophy in an accelerated mode as compared to human patients. The rapid disease progression in the porcine model is most likely associated with its high growth rate. Since fetal pig muscles grow by hypertrophy from around day 75 of pregnancy [55] and the mechanical strain per unit surface area increases with the caliber of muscle fibers [56], dystrophin deficiency

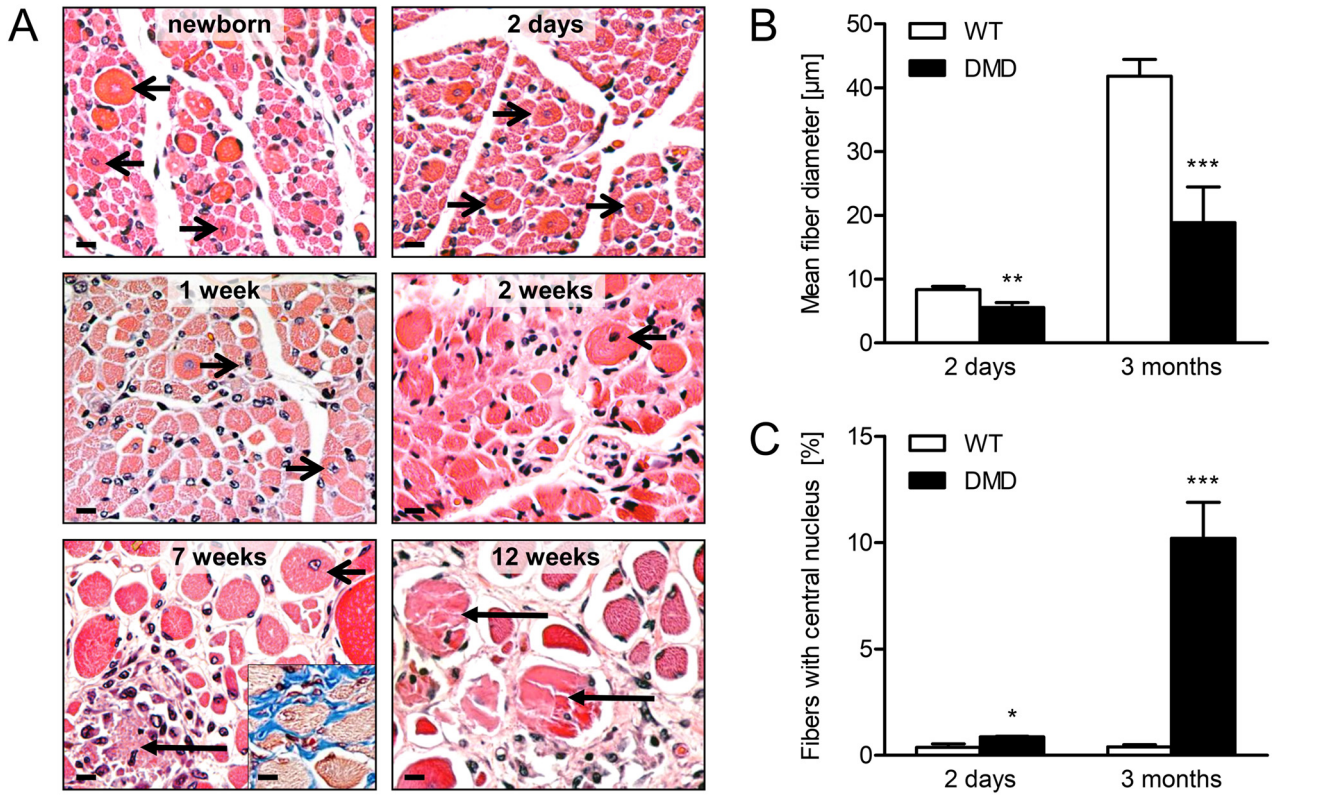

Fig. 3. Age-related progression of severity of structural alterations in skeletal muscle of DMD pigs. (A) Histology of the biceps femoris muscle, paraffin sections, hematoxylin and eosin (H&E)-staining; inset: demonstration of interstitial fibrosis by Masson's trichrome-staining (blue color). Short arrows indicate large, rounded fibers with internalised central nuclei, long arrows necrosis of muscle fibers. Bars = 10 µm (from [42]). (B) Mean minimal Feret's diameter of muscle fiber cross section profiles. (C) Proportion of muscle fiber cross section profiles with central nuclei. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$ (from [42]).

may render them particularly susceptible to sarcolemmal damage. This is supported by the concept that timing and pattern of muscle fiber growth influences the severity of dystrophic changes of DMD muscle [57].

4. Altered molecular profiles of skeletal muscles and myocardium of DMD pigs

As expected, *DMD*Δ52 mutant pigs are dystrophin deficient as shown by immunofluorescence and Western blot analyses [42]. As DMD patients and *mdx* mice (reviewed in [58]), DMD pigs revealed a compensatory upregulation of utrophin, which was moderate at age 2 days and marked at age 3 months [42]. However, in 2-day-old DMD piglets the utrophin signal was restricted to blood vessels, while in 3-month-old DMD pigs clear staining of the sarcolemma was observed. Similarly, young cases of DMD in humans show little sarcolemmal utrophin, but the level increases upon disease progression (reviewed in [59]). The lack of up-regulation of utrophin at the sarcolemma in 2-day-old DMD piglets may contribute to the high rate of early mortality. Similarly, utrophin immunostaining was absent, faint or variably positive in skeletal muscle samples from 2-day-old dystrophic CXMD puppies which died spontaneously [60].

Standardized tissue repositories of DMD and WT control pigs at different disease stages represent a unique resource for holistic molecular profiling studies gaining insight into pathomechanisms of DMD.

Transcriptome profiling of skeletal muscle samples from 2-day-old and 3-month-old DMD pigs produced by cloning and age-matched controls provided first insights into the hierarchy of physiological derangements of dystrophic muscle [42]. The muscle transcriptome changes in 3-month-old DMD pigs were very similar to those reported for muscle samples of DMD patients [61] and reflected progressive muscular dystrophy involving degeneration, regeneration, inflammation and reactive fibrosis going along with a severe metabolic disturbance. In contrast, transcriptome profiling of muscle samples from 2-day-old DMD pigs did not reveal signatures pointing to extracellular matrix remodeling, inflammatory response or decreased energy metabolism. These transcriptome changes were more similar to acute exercise muscle injury [62].

A holistic proteome analysis of corresponding skeletal muscle samples using high-end mass spectrometry showed that the extent of proteome changes in DMD vs. WT muscle increased markedly with age, reflecting progression of the pathological changes [63]. In samples from 2-day-old DMD piglets, proteins related to muscle development and cytoskeleton organization were increased in abundance, whereas proteins involved in translation and glycolysis were less abundant than in WT samples. In 3-month-old DMD muscle, proteins related to muscle repair such as vimentin,

nestin, desmin and tenascin C were found to be increased, whereas a large number of respiratory chain proteins were decreased in abundance in DMD muscle, indicating serious disturbances in aerobic energy production and a reduction of functional muscle tissue. The combination of proteome data for fiber type specific myosin heavy chain proteins and immunohistochemistry showed preferential degeneration of fast-twitch fiber types in DMD muscle [63].

To get insights into molecular pathways affected during progression of DMD-associated cardiomyopathy, Tamiyakul, Kemter [64] performed a proteome analysis of myocardium samples from the same cohort of cloned 2-day-old and 3-month-old DMD pigs and corresponding WT controls. Compared to data from skeletal muscle, this study provided clear evidence that DMD progression in myocardium is not only slower than in skeletal muscle, but also involves different biological and biochemical pathways. Functional categories of proteins with higher abundance in 2-day-old DMD vs. WT piglets included 'regulation of protein complex assembly' and 'regulation of cytoskeleton organization', among them spectrin alpha 1 (SPTA1), spectrin beta chain (SPTB), and ankyrin-1 (ANK1). Additionally, several proteins directly linked to sarcomeres, like keratin 8 (KRT8), keratin 19 (KRT19), caveolin 3 (CAV3), and myotilin (MYOT), were found to be differentially abundant. The most prominent cluster of proteins decreased in abundance in 2-day-old DMD piglets was 'translation' containing 35 ribosomal proteins. Additionally, several muscle related proteins, like syntrophin alpha 1 (SNTA1), myosin 6 (LOC100736765), myosin regulatory light chain 2 (MYL7), and the two creatine kinases (CKM and CKB) were found to be significantly reduced. In 3-month-old DMD pigs, the most prominent cluster of proteins with increased abundance was 'inflammatory response' including - among others - complement component C7 (C7), alpha-2-macroglobulin (A2M), protein S100-A8 (S100A8), and Toll-interacting protein (TOLLIP). Furthermore, similar to the 2-day-old animals, several proteins related to cardiac muscle contraction, i.e. KRT8, MYL7 and myosin light chain 4 (MYL4) were found to be higher abundant than in age-matched WT samples. Protein clusters with decreased abundance in 3-month-old DMD pigs were 'translation' and 'ribosomes'. In addition, the levels of several sarcoglycans (SGCA, SGCB and SGCD) and dystrobrevin alpha (DTNA) were decreased [64] in 3-month-old DMD animals.

These age-related proteome changes in skeletal muscle and myocardium were largely reflected in the analyses of samples from *DMD*$^{Y/-}$ and WT pigs produced by breeding [44].

For this review, we performed a meta-analysis - statistical aggregation of independent research results - to summarize proteome alterations induced by DMD in skeletal muscle [44,63,65] and myocardium [44,64] at different ages (Fig. 4A). Specifically, we applied the Stouffer's method (implemented in MetaMSD package [66,67] to combine significance (p-value) of proteome alteration associated with the lack of functional dystrophin (Table S1). The meta-analysis clearly demonstrates substantial proteome changes in both tissues and age groups. In accordance with our previous findings [44] and the human pathology, the meta-analysis demonstrates that the skeletal muscle is the most affected type of muscle in DMD (Fig. 4B). Similar to our previous findings, the meta-analysis revealed a more pronounced dysregulation of proteins involved in energy production via oxidative phosphorylation in skeletal muscle (Fig. 4C) compared to myocardium. Furthermore, the previously described inverse dysregulation of multiple ribosomal proteins (involved in translation) was found in skeletal muscle and myocardium (Fig. 4D).

5. Validation of multispectral optoacoustic tomography (MSOT) as an imaging biomarker for monitoring DMD progression

Duchenne patients are usually first diagnosed at an average age of 4 years [68,69]. For monitoring disease progression and effects of therapeutic interventions, pediatricians need diagnostic procedures, which are fast, repeatable, non-invasive, and independent of the patient's cooperation or current performance. Up to date, the six-minute walk test is still one of the most relevant parameters in DMD diagnostics and an important parameter in clinical trials [70], although it depends on motivation and the remaining ability to walk and finally carries the risk to fall, if the motivation is stronger than the remaining muscle strength.

Magnetic resonance imaging (MRI) of the muscle has been proven as an objective, reproducible, noninvasive method of quantifying dystrophic abnormalities, including changes in edema, fat infiltration, muscle volume, and tissue composition, and in particular fatty degeneration. A recent systematic review also found moderate to excellent correlations between MRI measurements and motor function [71]. However, long acquisition times with the requirement of sedation in early childhood and uncooperative patients limit the routine practical application of MRI.

Multispectral optoacoustic tomography (MSOT) is an imaging modality that allows quantitative in vivo detection of specific biomolecules in tissue. In general, optoacoustic imaging combines optical illumination and ultrasound detection. A nanosecond pulsed laser (pulse duration < 10 ns) in the near-infrared spectrum (680 to 1100 nm) is used to illuminate the tissue. Absorption by different molecules in the tissue converts the optical energy into heat, and the resulting thermoelastic expansion generates acoustic waves which can be detected by an ultrasonic transducer and converted to an image (reviewed in [72]). Since acoustic waves are much less scattered in tissue than light, optoacoustic imaging can generate high-resolution optical contrast images in biological tissues up to centimeters depths [73]. Moreover, since different tissue chromophores, such as deoxy- and oxy-hemoglobin, melanin, lipid, collagen, and water, have characteristic absorption spectra, spectral unmixing allows the relative quantification of these molecules.

Since progressive muscle fibrosis is a pathological hallmark of DMD, Regensburger, Fonteyne [65] attempted to quantify collagen in muscle tissue by MSOT. Due

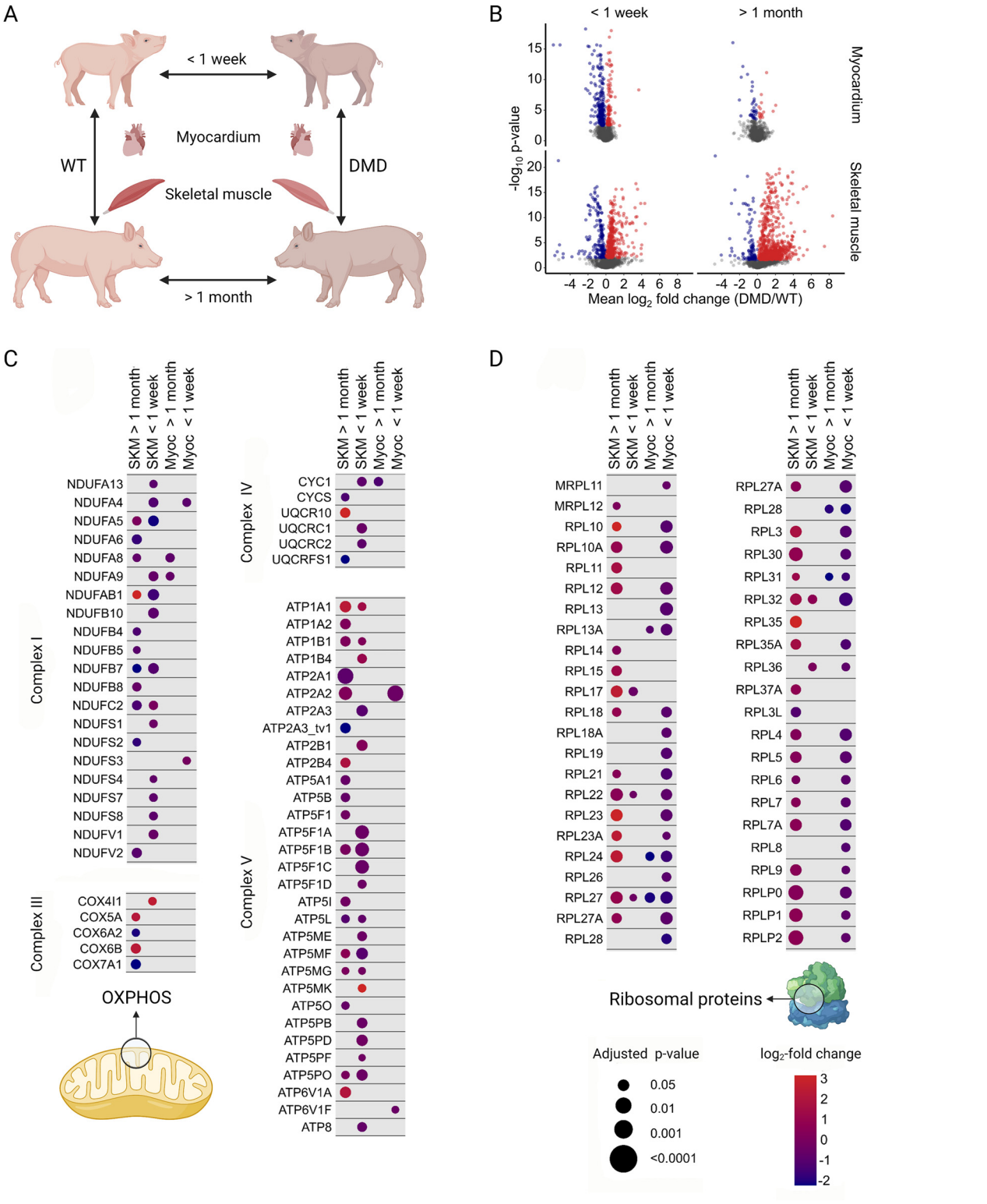

Fig. 4. Meta-analysis of the proteomic datasets. (A) Overview of the experimental strategy. (B) Meta-analysis volcano plots of differentially abundant proteins from myocardium (top row) and skeletal muscle (bottom row) for < 1 week (left column) and > 1 month (right column) DMD pigs. For the meta-analysis, p-values from DMD proteomics datasets were combined for each tissue type and age group using the Stouffer's method. The Bonferroni method was used for p-value adjustment. Proteins significantly altered in abundance (false discovery rate < 0.01) in DMD are colored in blue and red for downregulation and upregulation respectively. (C, D) Bubble plot of proteins related to oxidative phosphorylation (C) and ribosomal translation (D) in myocardium and skeletal muscle at different ages from DMD pigs. Protein alterations in DMD versus WT are color-coded and depicted as log2 fold-change of DMD/WT. Circle size corresponds to the significance of the change. Only proteins with false-discovery rate < 0.05 in at least one comparison are shown. SKM, skeletal muscle, Myoc, myocardium, OXPHOS, oxidative phosphorylation. Created in part with BioRender.com.

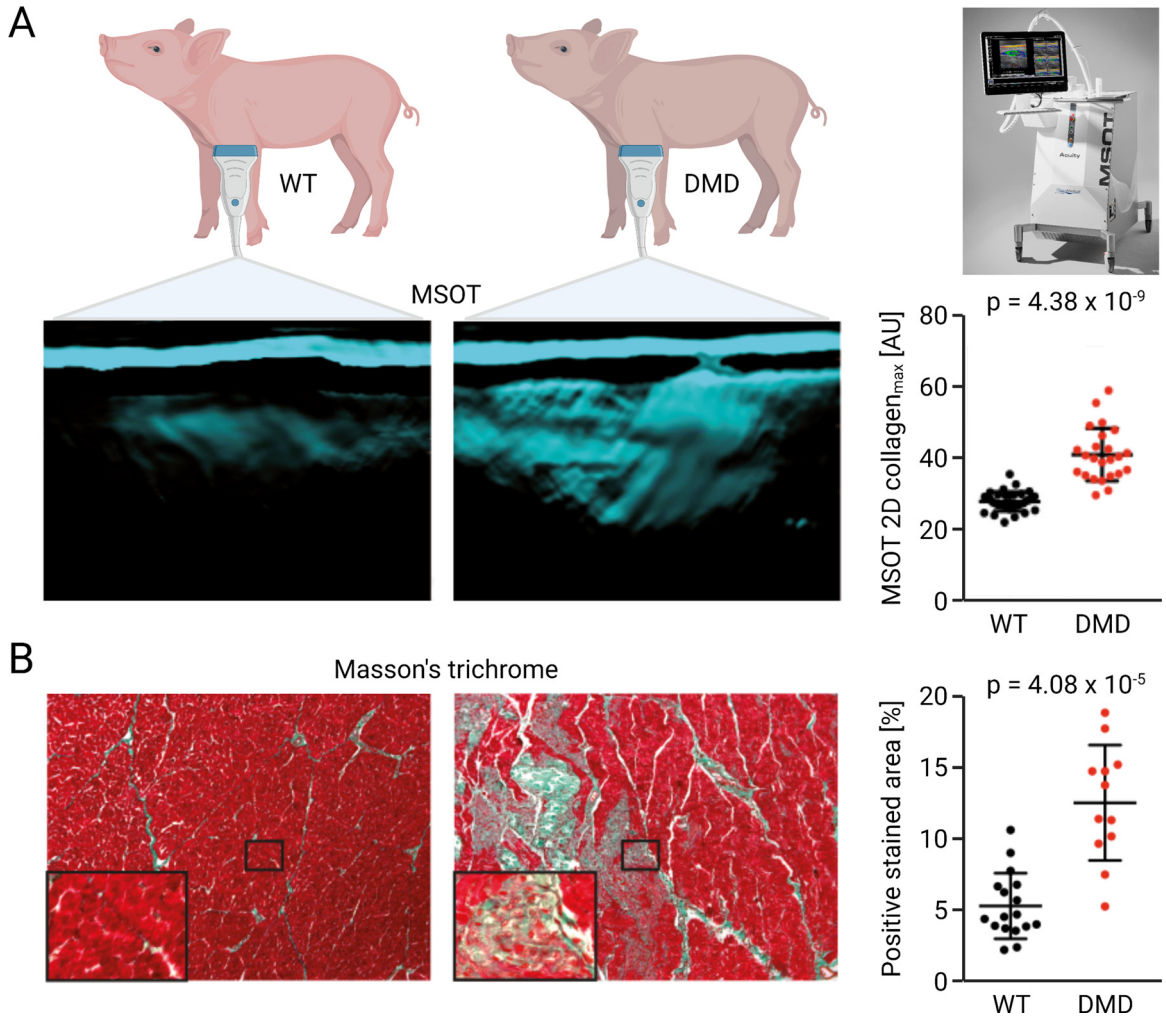

Fig. 5. Multispectral optoacoustic tomography (MSOT) revealing progressive fibrosis of skeletal muscles in $DMD^{Y/-}$ pigs. (A) MSOT scans and (B) histological quantification of fibrosis (Masson's trichrome staining) correlated well for each group but differed significantly between $DMD^{Y/-}$ and age-matched WT piglets (modified from [65]). Created with BioRender.com.

to the rapid progression of muscular dystrophy, $DMD^{Y/-}$ piglets represented an ideal model for the validation of this technology (Fig. 5). After establishing the parameters for collagen detection by MSOT, a longitudinal study of biceps femoris and triceps brachii muscles of $DMD^{Y/-}$ piglets and age-matched WT controls was conducted ($n = 4$ investigations in weekly intervals starting in 1- to 3-day-old animals). While quantitative MSOT-derived collagen signals in WT piglets remained constant during the experiment, the MSOT-derived collagen signals of muscles of $DMD^{Y/-}$ piglets increased steadily reaching a difference of 1.5- to 1.7-fold compared to WT after 4 weeks. Increased collagen deposition was confirmed by histological staining (Masson's trichrome, Sirius red), hydroxy proline quantification, and detection of specific collagens by mass spectrometry, thus establishing a quantitative correlation of MSOT-derived collagen signals with actual muscle fibrosis [65].

A first-in pediatric trial of MSOT enrolling DMD patients ($n = 10$) and age- and gender-matched healthy volunteers ($n = 10$) showed similar increased signals in different muscle regions of DMD patients. Furthermore, significant negative correlations between MSOT-derived collagen signals and standard clinical tests for DMD disease progression could be established, qualifying MSOT in the extended near-infrared range as a clinically meaningful modality for non-invasive in vivo detection of collagens and disease progression in DMD [65].

6. Somatic gene editing to restore an intact *DMD* reading frame in DMD pigs

Excision of the mutated *Dmd* exon 23 in the *mdx* mouse model using CRISPR/Cas9 – either by zygote injection [74] or by AAV-mediated somatic genome editing in muscle cells [75–77] – restores an intact *Dmd* reading frame, leading to the synthesis of an internally shortened but functional dystrophin protein. However, for a number of reasons the translation of this exciting concept from mouse models to human patients is challenging. Open questions are efficient delivery in a three orders of magnitude larger organism with a much more severe muscular pathology compared to the

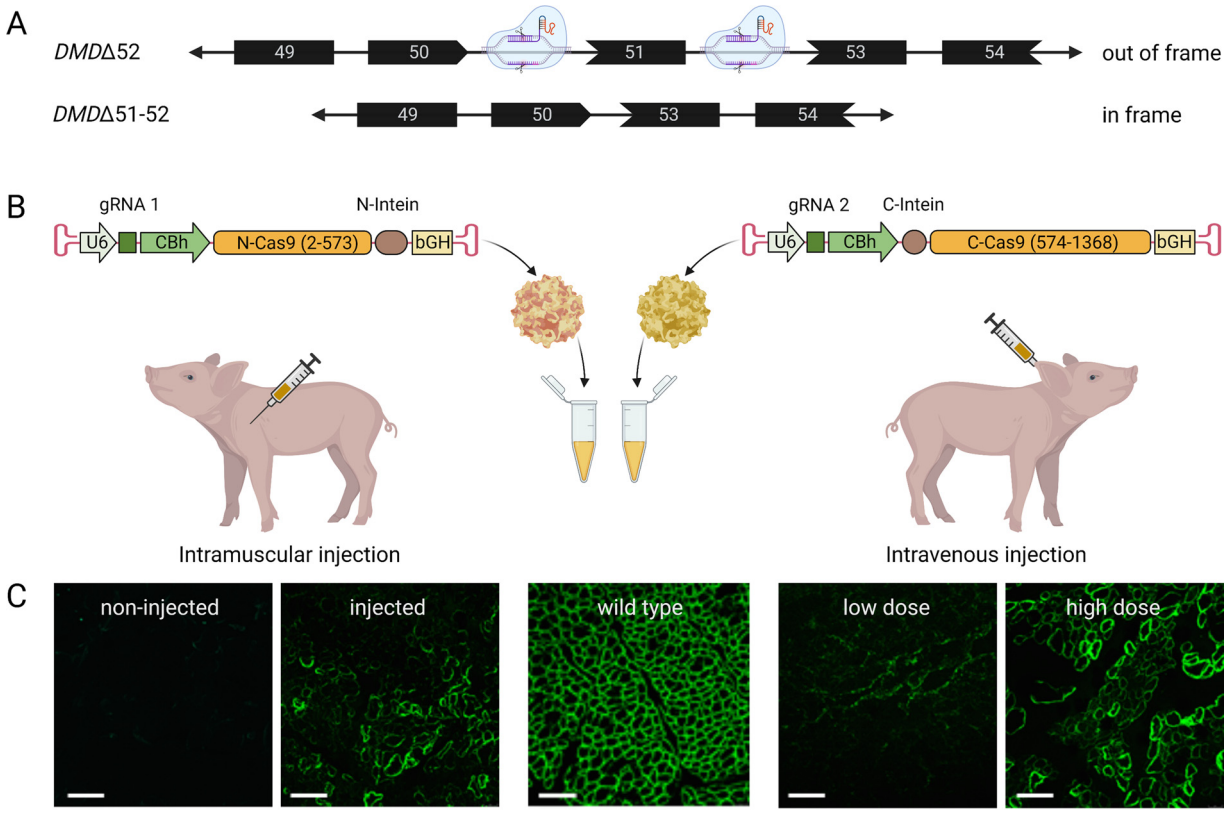

Fig. 6. Somatic gene editing to restore in-frame *DMD* transcripts in $DMD^{Y/-}$ pigs. (A) Correction of the frameshift mutation in the *DMD*Δ52 pig model by additional deletion of exon 51, resulting in an in-frame mutation (DMDΔ51–52). (B) AAV9 vectors for excision of *DMD* exon 51. The first AAV vector carries the information for the N-terminal part of the Cas9 protein and the first guide RNA (gRNA), targeting a specific sequence upstream of exon 51. The second contains the sequences for the C-terminal part of Cas9 and the second gRNA with a targeting sequence downstream of exon 51. Viral particles with the first and the second vector were injected subsequently. First pigs were treated locally by intramuscular injection, in another collective of animals low-dose and high-dose systemic (intravenous) application was tested. (C) Immunofluorescence staining specific for dystrophin of skeletal muscle showed total absence of dystrophin in untreated $DMD^{Y/-}$ animals, but high expression in muscle fiber membranes of wild-type pigs. Local injected animals present only local dystrophin expression, while all explored muscles of systemic injected piglets expressed moderate (low dose) to high (high dose) levels of dystrophin (only biceps femoris muscle is illustrated; modified from [49]). Created with BioRender.com.

mdx mouse, the optimum timing of the intervention, and the minimum level of dystrophin required for a therapeutic benefit, to name but a few. Therefore, preclinical studies in clinically severe large animal models are crucial for the clinical translation of targeted therapies, such as gene editing.

In the *DMD*Δ52 pig model, which resembles one of the most frequent *DMD* mutations in humans (reviewed in [1]), an intact reading frame can be restored by additional deletion of exon 51 (or exon 53). Therefore, Moretti, Fonteyne [49] selected an efficient pair of guide RNAs upstream and downstream of *DMD* exon 51 and expressed them from 2 different AAV9 vectors together with the N-terminal or C-terminal part of an intein-split *Streptococcus pyogenes* Cas9 nuclease [78], respectively (Fig. 6). Six weeks after unilateral intramuscular injection of 2×10^{13} viral particles (vp) for each vector per kg body weight (BW) into 10- to 14-day-old piglets, histology revealed restitution of membrane-localized dystrophin in the treated areas, and – due to leakage of the vector – in low levels at the contralateral location. In addition, intravenous (i.v.) application of the AAV9 particles was tested. Therefore, the viral particles were coated with polyamidoamine dendrimer (PAMAM-G2) nanoparticles to increase their myotropism without enhancing toxicity. While after application of a low dose ($1–2 \times 10^{13}$ vp for each vector/kg BW) only sporadic transduction of skeletal muscle cells was observed, a high dose (2×10^{14} vp for each vector/kg BW) enabled dystrophin protein expression in skeletal muscles, diaphragm and heart. In skeletal muscle tissue, up to 26% *DMD* gene editing was achieved. Clinical benefits of the high-dose i.v. treatment included an improved activity profile of the DMD piglets as well as a reduction of serum creatine kinase activities [49]. In addition, structural improvements of the skeletal muscle tissue, such as a reduced occurrence of rounded myofibers with centralized nuclei, an increased capillary density as well as decreased mononuclear cell infiltration and interstitial fibrosis were observed. Augmented muscle twitch amplitude and tetanic contraction force indicated functional improvement after high-dose treatment.

While the reduction in left ventricular (LV) ejection fraction in *DMD*Δ52 pig hearts was not significantly improved by the high-dose AAV9 treatment, the decreased

voltage amplitude uncovered by electrophysiological mapping and caused by fibrosis in ventricles of untreated *DMD*Δ52 animals was positively affected. Moreover, analysis of single cardiomyocytes in 300-μm heart slices from high-dose i.v. AAV9-treated vs. untreated *DMD*Δ52 pigs showed a positive effect of the treatment on intracellular Ca^{2+} and possibly an amelioration of the inherent arrhythmogenic susceptibility of *DMD*Δ52 cardiomyocytes [49].

Restoration of dystrophin and improvements of disease mechanisms by Cas9-mediated excision of exon 51 in the *DMD*Δ52 pig model and in induced pluripotent stem cell (iPSC)-derived myoblasts and cardiomyocytes from a corresponding DMD patient [49] pave the way towards clinical translation of this novel treatment approach.

In addition to gene editing, *DMD*Δ52 pigs proved also to be useful for the optimization of oligonucleotide-based exon skipping strategies. Echigoya and coworkers designed several potential antisense oligonucleotides, precisely phosphorodiamidate morpholino oligomers (PMOs), against exon 51 or 53 and tested five of them for each exon with regard to their efficiency for exon skipping and thus to restore the reading frame in primary skeletal muscle cells of their *DMD*Δ52 pig model [45]. While frame-shift correction was detected by RT-PCR analysis for both target exons, dystrophin expression could be demonstrated by Western blotting only for the exon 51 skipping approach. Additionally, this study showed that conjugation of PMOs to cell-penetrating peptides (CPP) increases the efficiency up to 20- to 40-times compared to unconjugated PMOs.

7. Conclusions and perspectives

Porcine DMD models substantially expand the spectrum of model organisms available for translational DMD research. Since DMD pigs can now be propagated by breeding, they are available for preclinical studies in statistically relevant numbers. Furthermore, the rapid disease progression, in particular the early cardiac phenotype, offers efficacy readouts of new therapies within reasonable time periods. Therapeutic concepts with positive preclinical outcomes in the clinically severe DMD pig model have a high chance to be also efficacious in DMD patients. Since genetic engineering/genome editing is now very well established in pigs, the best possible outcomes of targeted therapies, such as Cas9-mediated exon 51 deletion, can be reliably predicted, e.g., by generation of *DMD*Δ51–52 pigs. Cell-type specific monitoring of molecular changes, e.g., by single nuclei sequencing of skeletal muscle in different stages of disease progression [79], has the potential to uncover new pathomechanisms of muscular dystrophy. Porcine DMD models can also be interesting for unraveling the causes of cognitive impairments that are frequently observed in patients with dystrophinopathies [80]. Behavioral changes indicative of cognitive impairment were observed in dystrophin deficient pigs, and standardized biosamples from various brain regions are available for molecular studies [44]. In particular, it will be interesting to characterize the spatial abundance of the shorter dystrophin isoforms Dp140 and Dp71 that are expressed in the human central nervous system [81]. The pig brain is gyrencephalic and thus resembles the human brain anatomy, growth and development more closely than rodent brains do (reviewed in [82]). Pigs are therefore increasingly used in neuropathology studies (reviewed in [83]). Another important resource of the DMD pig breeding colony are the *DMD*$^{+/-}$carrier pigs, some of which also showed cardiac manifestation [44]. A dedicated biobank from these carrier animals including blood, urine, and tissue samples will be useful for the discovery of biomarkers predicting the risk of a severe phenotype offering the possibility of early therapeutic intervention.

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Author contributions

M.S. and E.W. wrote the initial draft of the manuscript. All authors revised the manuscript (model development: L.M.F., B.K., M.K., V.Z., H.N., N.K.; pathology: A.B., E.K., K.M., M.C.W., R.W.; molecular profiling: B.S., J.B.S., G.J.A., H.B., T.F.; imaging: F.K.; therapy: M.C.W., W.W., C.K.) and approved the final version of the manuscript.

Declarations of Competing Interest

None

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