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Trends in
Molecular Medicine

Review

Pig models for translational Duchenne muscular dystrophy research

Michael Stirm [1,2,3], Nikolai Klymiuk [2,4], Hiroshi Nagashima [5], Christian Kupatt [6], and Eckhard Wolf [1,2,3,*]

Duchenne muscular dystrophy (DMD) is caused by mutations in the X-linked *DMD* gene, resulting in the absence of dystrophin, progressive muscle degeneration, and heart failure. Genetically tailored pig models resembling human *DMD* mutations recapitulate the biochemical, clinical, and pathological hallmarks of DMD with an accelerated disease progression compared to human patients. DMD pigs have been used to evaluate therapeutic concepts such as gene editing to reframe a disrupted *DMD* reading frame or the delivery of artificial chromosome vectors carrying the complete *DMD* gene. Moreover, DMD pigs have been instrumental in validating new diagnostic modalities such as multispectral optoacoustic tomography (MSOT) for non-invasive monitoring of disease progression. DMD pigs may thus help to bridge the gap between proof-of-concept studies in cellular or rodent models and clinical studies in patients.

Highlights

Pigs can be genetically engineered to resemble human monogenic diseases.

Genetically tailored pig models may bridge the gap between proof-of-concept studies in cellular or rodent models and clinical trials.

DMD pigs show the characteristic biochemical, clinical, and pathological features of DMD, with an accelerated development of the disease.

DMD pigs provide new insights into the hierarchy of physiological abnormalities of dystrophic muscle.

DMD pigs are a promising model for testing targeted therapies, such as exon skipping and gene editing, as well as for validating and optimizing novel diagnostic procedures such as MSOT.

Monogenic diseases and targeted therapies

Rare diseases are characterized by their low occurrence in the general population, and typically affect fewer than 50–60 individuals per 100 000. Monogenic diseases encompass a diverse range of clinical features and pathophysiological mechanisms, and >4000 monogenic mutations constitute at least 80% of all rare diseases [1]. Notably, these rare monogenic diseases often manifest in childhood and pose significant challenges in biology and medicine owing to their limited treatment options. Animal models are essential for identifying disease mechanisms and developing effective therapies. Although the development of mouse models is straightforward [2], large animal models are additionally required for translational studies [3]. This is exemplified by small and large animal models of DMD which have evolved as a paradigm for the development of targeted therapies [4]. This review covers the pros and cons of genetically tailored pig models for monogenic diseases in general and for DMD in particular. DMD pigs have provided important insight into the molecular abnormalities of skeletal muscle and myocardium during disease progression. The translational potential of porcine DMD models was demonstrated by studies of targeted therapies, such as gene editing [5] and artificial chromosome transfer [6], as well as by the validation of new diagnostic tools such as MSOT [7]. Moreover, systematic studies of heterozygous *DMD* mutation carriers have revealed a broad range of alterations in skeletal muscle and heart, depending on the abundance of dystrophin [8,9]. In addition, DMD pigs may be used for testing therapies based on *DMD* mini- or micro-genes [4], strategies for upregulating utrophin expression [10], and for the evaluation of *trans*- and *cis*-acting genetic polymorphisms affecting DMD disease progression and severity [11] (Figure 1). The rapid disease progression in porcine DMD models provides efficacy readouts in a reasonable time frame.

Monogenic disease modeling in pigs

Over the past three decades the pig has emerged as an attractive model organism for a wide range of medical research. An efficient toolbox for genetic engineering/gene editing (Box 1) is

[1]Chair for Molecular Animal Breeding and Biotechnology, Gene Center and Department of Veterinary Sciences, Ludwig Maximilian University of Munich (LMU Munich), 81377 Munich, Germany
[2]Center for Innovative Medical Models (CiMM), LMU, Munich, 85764, Oberschleißheim, Germany
[3]Interfaculty Center for Endocrine and Cardiovascular Disease Network Modelling and Clinical Transfer (ICONLMU), LMU Munich, 81377 Munich, Germany
[4]Large Animal Models in Cardiovascular Research, Internal Medical Department I, Technical University of Munich (TU Munich), 81675 Munich, Germany
[5]Meiji University International Institute for Bio-Resource Research, Kawasaki, Kanagawa 214-8571, Japan

available for the development of genetically tailored pig lines. Pigs share many anatomical and physiological similarities with humans, such as size, coronary distribution, and heart rate. As a consequence, the pig is the preferred source species for organ **xenotransplantation** (see Glossary) [12–14]. The advantages of the pig as a biomedical model include its high fecundity, fast maturation (6 months), short gestation period (114 days), breeding under specified pathogen-free (SPF) conditions, and fewer ethical restrictions compared to nonhuman primates or dogs [15]. Furthermore, decades of experience in the care, breeding, and rearing of this significant livestock can be drawn upon [16]. Important resources such as reference genomes from multiple pig breeds [17–21], the pig BodyMap transcriptome database [22], innovative approaches for proteome [23,24] and metabolome research [25], specific tools for the analysis of complex traits [26] and the underlying genetic regulatory mechanisms [27], standardized tissue

[6]Klinik und Poliklinik für Innere Medizin I, Klinikum Rechts der Isar, TU Munich and German Center for Cardiovascular Research (DZHK), Munich Heart Alliance, 81675 Munich, Germany

*Correspondence
ewolf@genzentrum.lmu.de (E. Wolf).

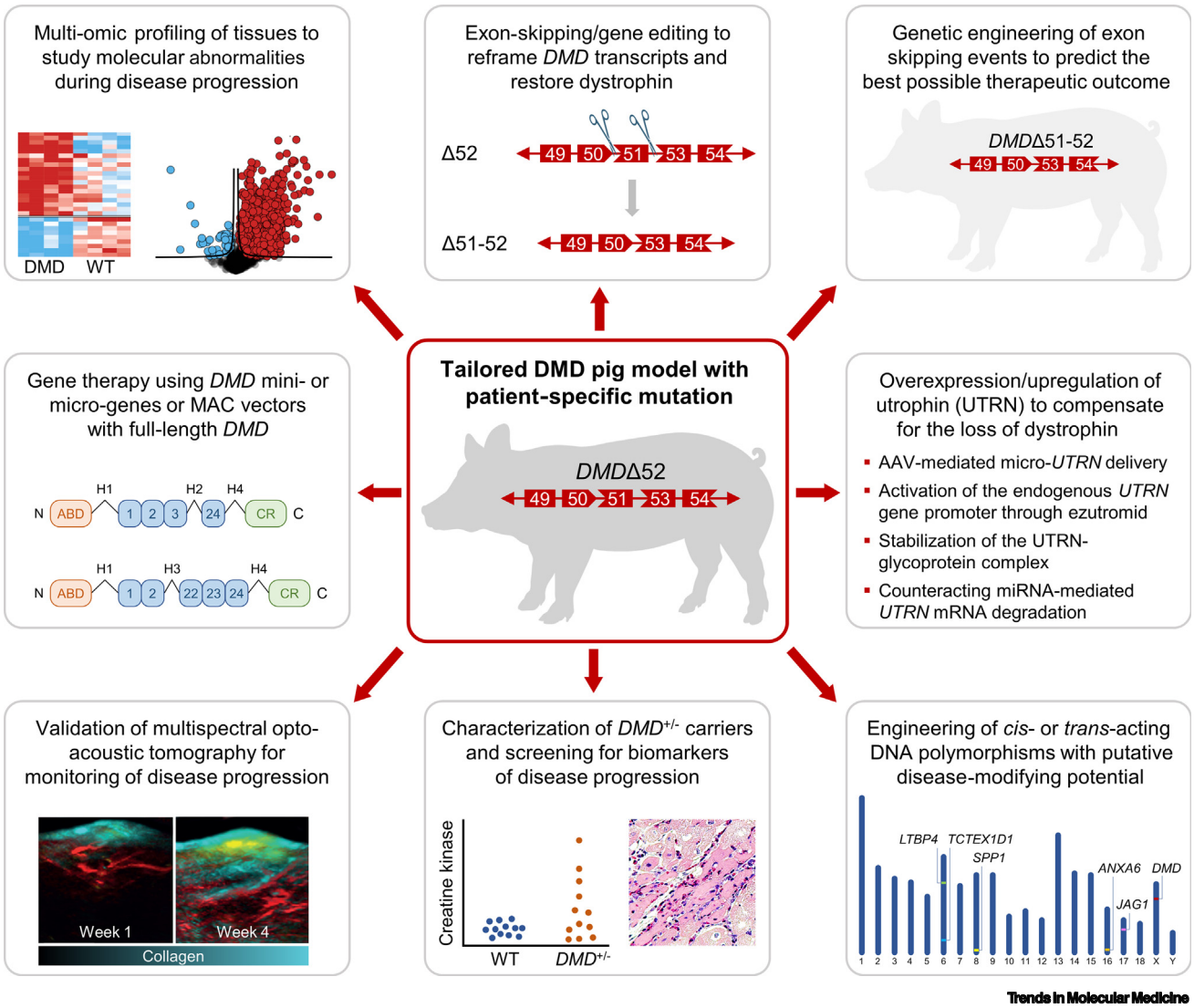

Figure 1. Current and possible future applications of pig models for Duchenne muscular dystrophy (DMD). Abbreviations: AAV, adeno-associated virus vector; ABD, actin-binding domain; ANXA6, annexin A6; CR, C-terminal region; JAG1, Jagged canonical Notch ligand 1; LTBP4, latent transforming growth factor β-binding protein 4; MAC, mammalian artificial chromosome; SPP1, secreted phosphoprotein 1; TCTEX1D1, Tctex1 domain containing 1; UTRN, utrophin; WT, wild type.

Box 1. Concepts for genetic modification (GM)/gene editing (GE) of pigs

Genetically modified pigs are currently mainly produced by somatic cell nuclear transfer (SCNT). The desired GM is introduced into cultured primary cells, which are then fused to enucleated oocytes to generate cloned embryos. After transfer to recipient pigs, the cloned embryos give rise to piglets harboring the GM originally introduced into the nuclear donor cells. The spectrum of GM includes additive transfer of gene constructs, transposon-mediated transgenesis, homologous recombination (HR) with 'classical' targeting vectors or modified bacterial artificial chromosome targeting vectors [66,101] with increased HR frequency [102]. GE using designer nucleases has revolutionized the development of porcine biomedical models. The induced DNA double-strand breaks can be repaired by non-homologous end-joining (NHEJ) or by HR, allowing the inactivation or targeted modification of specific genes, respectively. Initially, zinc-finger nucleases (ZFN) or transcription activator-like effector nucleases [103] with protein motifs targeting the nuclease to specific DNA sites were used. The RNA-guided clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) system is even more versatile and has been used to modify multiple loci in the pig genome in a single step. The CRISPR/Cas system can be delivered as a single guide RNA (sgRNA) and mRNA for Cas9 protein, as a plasmid encoding Cas9 and sgRNA, or as a Cas9/sgRNA ribonucleoprotein complex. The combination of CRISPR/Cas tools with targeting vectors also facilitates the placement of expression cassettes into safe harbor loci (such as *ROSA26*) and the creation of complex genetic modifications, such as the partial or even complete humanization of target genes, allowing preclinical testing of targeted therapies for patient-specific mutations (e.g., [104,105]). In addition, Cas9 transgenic pigs were developed as an important resource for *in vivo* gene editing studies [106–108] such as for organ-specific induction or correction of mutations.

GE components can also be injected into zygotes; however, there is a risk of mosaic formation (varying GE events in different cells of the resulting animal) that require a generation of breeding (1 year) to obtain animals with uniform gene edits.

Importantly, the timelines for generating new GM pig models are significantly longer than for mouse models, and the costs are accordingly much higher. GM founder animals can be obtained after 4–6 months. They become sexually mature after 6–8 months and require another 4 months to deliver offspring. Thus, the first animals for experimental studies may be available 15–20 months after the initial design of new GM pig model.

sampling protocols [28], and biobanks of dedicated pig models (e.g., [29]) have been established. Moreover, recent advances in porcine pluripotent stem cell research provide new cellular models and the basis for testing cell therapies [30–32]. The development of porcine models of monogenic diseases and their implementation for translational research is thus straightforward [33,34]. Nevertheless, there are important limitations to the widespread use of genetically modified (GM) pig models, including genetic variability within and between pig lines, limited availability of genetic resources such as Cre driver lines [35], the requirement for specialized facilities and high maintenance costs, and limited availability of validated laboratory tools and reagents such as antibodies.

DMD as a model genetic disease

DMD is a fatal neuromuscular disease caused by loss-of-function mutations in the X-linked dystrophin gene (*DMD*; ~2.2 Mb, 79 exons), and a major mutation hotspot is located in exons 45–55 [36]. Mutations of the *DMD* gene occur at a higher frequency than the typical mutation rate, leading to approximately one affected boy in every 5000–6000 male births [37]. Common *DMD* mutations include whole-exon deletions (68%), exon duplications (11%), and nonsense mutations (10%). Disruption of the *DMD* **open reading frame (ORF)** results in the complete absence of functional dystrophin, which connects the actin cytoskeleton via a large complex of dystrophin-associated glycoproteins to the extracellular matrix and serves primarily as a 'shock absorber' that protects muscle cells from contractile damage [4]. The absence of dystrophin leads to damage to muscle fibers, causing pathological calcium influx [38] and progressive muscle weakness, loss of ability to walk, reliance on artificial breathing support, and premature death from cardiac or respiratory failure [39].

DMD stands out as a pivotal focus in experimental therapeutics because of several factors: it represents an unmet clinical need marked by an early loss of ambulation, rapid disease progression towards respiratory and cardiac failure, and severely reduced life expectancy. Moreover, DMD is present at a higher incidence than other rare diseases, is intensely researched for molecular and

Glossary

Adeno-associated virus (AAV) vectors: small, non-enveloped viruses with a single-stranded DNA genome that are widely used in gene therapy; limitations include limited cargo capacity (~4.7 kb) and potential immune responses.
Creatine kinase (CK): catalyzes the conversion of creatine and ATP into phosphocreatine and ADP; increased CK activity in blood serum serves as a marker of muscle, heart, or brain damage.
Exogenesis: the development of human cells in animal hosts by complementation of organogenesis-impaired animal embryos with human stem cells.
Induced pluripotent stem cells (iPSCs): pluripotent cells derived from terminally differentiated somatic cells generated by reprogramming via the introduction of a defined set of transcription factors.
Magnetic resonance imaging (MRI): uses powerful magnets, radiofrequency waves, and computer processing to create detailed images of internal structures of the body. By aligning hydrogen protons in water molecules with a strong magnetic field, applying radiofrequency pulses, and detecting emitted signals, MRI produces high-resolution images, making it a valuable tool for visualizing soft tissues without using ionizing radiation.
Mammalian artificial chromosomes (MACs): engineered mini-chromosomes that serve as gene delivery vectors for large gene inserts, characterized by high stability in host cells independently of the host genome. MACs can be constructed through either a bottom-up approach, involving *de novo* assembly of alphoid DNA in host cells to create circular structures, or a top-down approach, where artificial chromosomes are engineered from natural chromosomes, resulting in linear structures with native centromere sequences.
Mesoangioblasts: vessel-associated stem cells that can differentiate into most mesodermal tissues.
Microcell-mediated chromosome transfer (MMCT): based on the fusion of target cells to microcells derived from chromosome donor cells. Chromosome donor cells are treated with colcemid which blocks spindle formation and induces the formation of micronuclei

cellular treatments, and has garnered recent regulatory approvals of targeted molecular therapies [4]. These include antisense oligonucleotides for exon skipping (Box 2) to reframe the *DMD* transcript, resulting in the production of an internally shortened but partially functional dystrophin protein. If achieved sufficiently early in the majority of muscle fibers and cardiomyocytes, this transformation may shift DMD towards Becker muscular dystrophy (BMD), which typically emerges later in life, displays milder symptoms, and progresses more slowly compared to DMD [37]. BMD patients, in contrast to DMD patients lacking dystrophin, have shorter or altered forms of the dystrophin protein (pseudo-dystrophin) resulting from in-frame *DMD* mutations, but may exhibit significant variations in the severity of their condition. This variability ranges from almost asymptomatic to severely affected, depending on the location and type of mutation within the *DMD* gene. This allows the preidentification of promising target sequences for exon-skipping strategies [40]. The exon-skipping treatment requires weekly intravenous oligonucleotide infusions because the reframing occurs at the pre-mRNA level. Four **phosphorodiamidate morpholino oligonucleotides (PMOs)** have been approved by the US Food and Drug Administration (FDA) for clinical use: eteplirsen for skipping of exon 51, viltolarsen and golodirsen for skipping of exon 53, and casimersen for skipping of exon 45 [4]. The levels of dystrophin restoration only ranged to a few percent of normal levels [41–43] and thus the clinical efficacy was limited. To improve the delivery of PMOs and the efficacy of exon skipping, conjugates of PMOs with cell-penetrating peptides [44] or antibodies/antibody fragments specific for the **transferrin receptor (TFRC)** that is highly expressed in skeletal and cardiac muscle cells [45] are in clinical development [4].

Other strategies to restore dystrophin in DMD include compounds that induce stop codon readthrough, gene therapy using **mini- or micro-dystrophin** expression vectors, and gene editing to induce changes resulting in an intact reading frame (i.e., removal of one or several exons or mutation of splice sites) [46]. The potential of this concept was demonstrated in the *mdx* mouse model of DMD that features a spontaneous nonsense point mutation in *Dmd* exon 23 and lacks dystrophin [27]. Removal of the mutated *Dmd* exon 23 using CRISPR/Cas9 – either in the germline through zygote injection [47] or by **adeno-associated virus (AAV) vector**-mediated somatic genome editing in muscle cells [48–50] – resulted in the production of a slightly shorter but functional dystrophin protein (Box 2).

Animal models of DMD

Animal models have played a crucial role in shaping these novel therapeutic concepts. In particular, the *mdx* mouse model has been pivotal in DMD research. In addition, various *mdx* mouse

containing one to several chromosomes.

Mini- or micro-dystrophin: internally truncated but functional pseudo-dystrophin proteins that typically lack large portions of the central rod domain but maintain the domains interacting with the cytoskeleton and the dystrophin-associated protein complex (DAPC).

Myogenic factor 5/6 (MYF5/6) and myogenic differentiation (MYOD/MYOD1): members of the myogenic regulatory factor family of transcription factors; their ablation in embryos prevents the formation of skeletal muscle.

Open reading frame (ORF): a contiguous non-overlapping set of triplet codons in DNA or RNA; the ORF contains the sequence from the start codon (AUG) to a stop codon (UAA, UAG, or UGA).

Phosphorodiamidate morpholino oligonucleotides (PMOs): short single-stranded DNA analogs that contain a backbone of morpholine rings connected by phosphorodiamidate linkages.

Transferrin receptor (TFRC): mediates the cellular uptake of iron from the plasma glycoprotein transferrin. Because of its widespread expression, TFRC is often used as target for drug delivery.

Tumor protein P53 (TP53): a transcription factor involved in DNA repair, senescence, cell-cycle control, autophagy, and apoptosis that acts as tumor suppressor.

Xenotransplantation: the transplantation of cells, tissues, or organs between different species.

Box 2. Exon skipping and gene editing for correcting disrupted *DMD* reading frames

The primary approach for restoring dystrophin in DMD is through exon skipping, aiming to correct the *DMD* reading frame and produce an internally shortened, partially functional pseudo-dystrophin protein. Single-stranded antisense oligonucleotides (ASOs; ~20–30 nt) are designed to interact with pre-mRNA transcripts through Watson–Crick base pairing and mask specific splicing signals, including exon splicing enhancers and exon recognition sequences. Although exon skipping is primarily useful for patients with complete exon deletions, it can also be applied to nonsense mutations if they occur within exons that can be skipped without disrupting the reading frame. Exon-skipping approaches may also be effective for some types of exon duplication. It is estimated that exon-skipping approaches may be relevant for 55% of DMD-causing mutations and 80% of DMD-causing deletions [4].

Gene editing is conceptually similar to exon skipping, but the correction of the reading frame is done at the DNA rather than the pre-mRNA level, resulting in a 'permanently exon-skipped' *DMD* locus. A CRISPR/Cas9 system with two sgRNAs is designed to induce double-strand breaks in the intronic sequences flanking the target exon. AAVs are used for systemic or intramuscular delivery. Multiplex gene editing allows simultaneous deletion of multiple exons, resembling multi-exon skipping, and offers a potential single therapy for a significant patient population. An alternative is the single-cut strategy where targeted introduction of an indel disrupts splicing motifs or reframes the transcript [46].

strains carrying patient-relevant mutations, such as *Dmd*Δ52, have been developed via gene targeting (reviewed in [51]). Severe pathological changes in skeletal muscle were only present in aged (88 weeks) mice [52], and fibrotic lesions in the myocardium were only found in exercised animals [53]. Moreover, the lifespan was only marginally reduced. Therefore, additional genes such as 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*) were mutated to aggravate the phenotype of *mdx* mouse models [51]. Nevertheless, large animal models are also imperative for translational DMD research. In addition to the original golden retriever muscular dystrophy (GRMD) dog model [54] and the beagle-based canine X-linked muscular dystrophy in Japan (CXMDJ) [55], various other lines with spontaneous canine X-linked muscular dystrophy have been reported [56,57]. In the Cavalier King Charles spaniel model of muscular dystrophy (CKCS-MD), that lacks splicing of exon 50 into the transcript, additional skipping of exon 51 can restore an intact *DMD* reading frame [58], making this model suitable for evaluating therapeutic approaches that are potentially beneficial for a substantial proportion of DMD patients. Despite the successful use of canine DMD models in preclinical studies [59,60], their use in experiments raises ethical concerns and poses challenges in breeding and maintenance [61]. In addition, DMD models have been created by gene editing in several other species, including rabbit [62], rat [63,64], and rhesus monkey [65]. In this review we focus on pig models of DMD which complement the existing models in other species, particularly for translational DMD research.

Porcine DMD models

The first porcine model of DMD was created using somatic cell nuclear transfer (SCNT) from male cells engineered to lack *DMD* exon 52 [66], a frequent mutation in human *DMD* [36]. The resulting cloned *DMD*Δ52 ($DMD^{Y/-}$) pigs lacked dystrophin and displayed reduced mobility and highly increased serum **creatine kinase (CK)** levels, indicating muscle damage that was evident from birth. Pathological analysis of skeletal muscles revealed severe and rapidly progressive muscular dystrophy with a maximum lifespan of 3.5 months. This precluded the propagation of the model by breeding (pigs reach sexual maturity at 5–7 months).

One option to overcome this limitation is to complement male *DMD*Δ52 embryos with blastomeres from female embryos with intact *DMD* alleles [67]. In the resulting intersex chimeric offspring, the muscular dystrophy was partially rescued by *DMD*-intact female cells, whereas the germline was derived from the male cells, enabling transmission of the *DMD*Δ52 allele to offspring.

The most sustainable approach for the establishment of a breeding colony of DMD pigs was the generation of a heterozygous carrier female ($DMD^{+/-}$) pig which could be used for breeding with wild-type (WT) boars to yield male $DMD^{Y/-}$, female $DMD^{+/-}$, and male and female WT offspring at the expected proportion of 25% each. The majority of DMD pigs generated in this way survive for 3–4 months, but they can exceptionally have a lifespan of 9 months [8].

Other *DMD*Δ52 pigs were produced by AAV-mediated gene targeting in male Yucatan miniature pig cells and subsequent SCNT [68]. In addition, attempts to generate Bama miniature pigs with mutations in *DMD* exon 51 [69] and Diannan miniature pigs with mutations in *DMD* exon 27 [70] have been reported.

Clinical and pathological investigations have shown that dystrophin-deficient pigs exhibit accelerated progression of muscular dystrophy in comparison to human patients [8,66]. The rapid muscle growth in pigs by hypertrophy of muscle fibers and the resulting mechanical strain on the sarcolemma may offer a potential explanation for the faster development of muscular dystrophy

compared to DMD in humans [15]. A particular advantage of the porcine DMD models is the early impairment of cardiac function, and a significant reduction of left ventricular ejection fraction is already present in 4-month-old *DMD*$^{Y/-}$ pigs [8]. Therefore, the efficacy of targeted therapies can be tested in porcine DMD models in a reasonable time frame. Disease-stage specific multiomic profiles of skeletal muscle [8,66,71] and myocardium samples [8,72] have provided important molecular readouts for studying disease progression and monitoring the efficacies of therapeutic interventions [5].

Therapeutic exon skipping and gene editing in *DMD*Δ52 pig models

Promising approaches to restore dystrophin face challenges when transitioning from mice to humans owing to issues such as the effective delivery of this treatment into a much larger organism with more severe muscle problems than those in the *mdx* mouse models. Questions remain about the best timing for intervention and the minimum level of dystrophin needed for therapeutic benefit. To address these concerns, preclinical studies using large animal models that closely mimic the severe conditions seen in human patients are essential.

In the *DMD*Δ52 pig model, the *DMD* transcript can be reframed by deleting exon 51 or exon 53. Primary skeletal muscle cells from a *DMD*Δ52 pig model [68] were used to test five PMOs for each exon to gauge their effectiveness in exon skipping and restoring the correct reading frame. Although both target exons showed frameshift correction, only exon 51 skipping resulted in dystrophin expression. Moreover, the study highlighted that linking PMOs to cell-penetrating peptides significantly enhanced their efficiency, and showed a 20–40-fold increase compared to unconjugated PMOs.

A gene editing approach to restore dystrophin in *DMD*Δ52 pigs used effective guide RNAs in conjunction with a *Streptococcus pyogenes* split-Cas9 nuclease expressed from separate AAV9 vectors to target *DMD* exon 51 [5]. Injecting these viral particles into the muscles of young piglets led to the recovery of dystrophin in the treated areas, and to some extent in other locations owing to vector leakage. For intravenous application the AAV9 particles were coated with polyamidoamine dendrimer nanoparticles to boost their muscle-targeting ability without causing harm [73]. Low doses showed minimal muscle cell transformation, but high doses resulted in substantial dystrophin production in muscles, diaphragm, and heart, reaching up to 32% gene editing in skeletal muscle tissue and >25% of WT dystrophin protein levels in the tibialis cranialis muscle. The high-dose intravenous treatment had a moderate but significant effect on the survival of DMD piglets (maximum lifespan 136 days vs 105 days in untreated animals) and revealed clinical improvements such as enhanced activity levels of the pigs and reduced serum CK activity. It also showed structural enhancements in muscle tissue, such as fewer abnormal myofibers, increased capillary density, and reduced immune cell infiltration and fibrosis. Muscle strength measurements indicated functional improvement post high-dose treatment [5].

AAV9-mediated *DMD* editing had a positive impact on the reduced voltage amplitude caused by fibrosis in untreated *DMD*Δ52 pig hearts. In addition, examination of individual cardiomyocytes from the hearts of pigs treated with high-dose intravenous AAV9 compared to untreated animals showed a beneficial effect on intracellular Ca^{2+} levels, potentially reducing the inherent susceptibility of *DMD*Δ52 cardiomyocytes to arrhythmias [5].

Modeling the best possible therapeutic outcome of gene editing

Because the efficacy of CRISPR/Cas9 and guide RNA delivery via AAVs into skeletal muscle cells and cardiomyocytes has limitations, the full therapeutic potential of *DMD* exon 51 deletion in *DMD*Δ52 pigs remained unclear in that approach. Therefore, *DMD* exon 51 was deleted by

gene editing in cells from *DMD*Δ52 pigs *in vitro*, and the *DMD*Δ51–52 cells were used for SCNT to generate pigs with the *DMD* frameshift correction in every cell [74] (Figure 2A). Skeletal muscle and myocardium samples from *DMD*Δ51–52 pigs exhibited positive dystrophin staining and lacked the dystrophic alterations typically observed in *DMD*Δ52 pigs (Figure 2B). Western blot analysis confirmed the presence of dystrophin in *DMD*Δ51–52 pigs, contrasting with its absence in *DMD*Δ52 pigs (Figure 2C). The proteome profile of skeletal muscle, that was marked by numerous abundance alterations in *DMD*Δ52 compared to WT samples, was normalized in *DMD*Δ51–52 samples. In accordance with this, the serum CK levels of *DMD*Δ51–52 pigs were in the physiological range (Figure 2D). At 3.5 months of age – when a contemporary cohort of *DMD*Δ52 pigs showed significantly reduced cardiac function – *DMD*Δ51–52 pigs exhibited complete rescue, aligning with the normalization of the myocardial proteome profile. These findings suggested that systemic deletion of *DMD* exon 51 in *DMD*Δ52 pigs substantially rescues the severe muscular dystrophy and impaired cardiac function seen in this model. Long-term monitoring of *DMD*Δ51–52 pigs will clarify whether the internally shortened dystrophin is fully functional and whether the animals develop characteristics of the milder BMD.

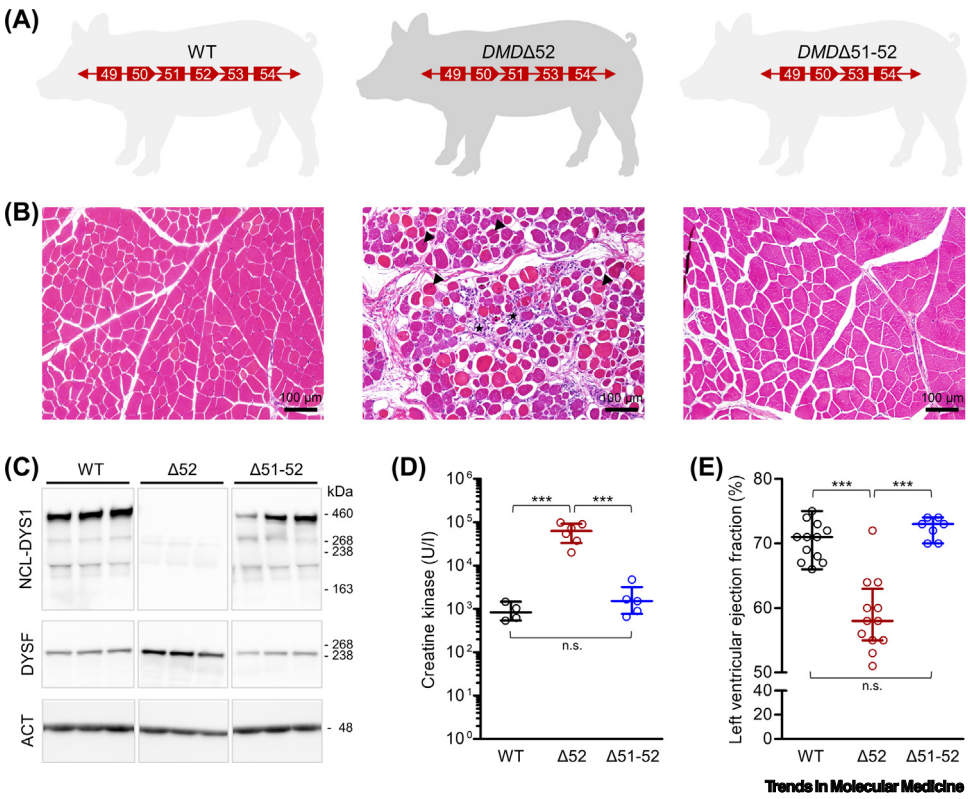

Figure 2. Modeling the best possible outcome of exon 51 deletion in Duchenne muscular dystrophy (DMD) caused by loss of *DMD* exon 52. (A) The wild-type portion (exons 49–54) of the *DMD* gene (left), out-of-frame deletion of exon 52 to create a model for DMD (middle), and in-frame deletion of exons 51 and 52 predicting the best possible outcome of exon 51 deletion in DMD caused by loss of exon 52 [74]. (B) Histology of triceps brachii muscle from 3.5-month-old pigs (hematoxylin-eosin staining). Arrowheads indicate muscle fibers with central nuclei, asterisks label inflammatory infiltration (panel adapted, with permission, from [74]). (C) Western blot analysis of dystrophin (NCL-DYS1), dysferlin (DYSF), and actin (ACT) in protein extracts from triceps brachii muscle (panel adapted, with permission, from [74]). (D) Serum creatine kinase (CK) activity and (E) left ventricular ejection fraction in 3.5-month-old pigs (panel modified, with permission, from [74]). Symbols and abbreviations: ***, P <0.001; n.s., not significant; U, units; WT, wild type.

Correction of DMD by artificial chromosome transfer

Because of the large size of the *DMD* gene (~2.2 Mb) and the limited capacity of viral vectors, gene therapy approaches for DMD cannot transfer the entire *DMD* gene and instead rely on constructs encoding mini- or micro-dystrophins [4,75]. Dystrophin minigenes lack non-essential internal domains that mimic the internally truncated but functional pseudo-dystrophin proteins of mildly affected patients. In micro-dystrophins the deletion is further extended to lack large portions of the central rod domain and often the C-terminal domain [4]. AAV-mediated delivery of micro-dystrophin constructs attenuated the dystrophic changes in murine [76] and canine DMD models [77,78]. Moreover, five micro-dystrophin drugs are currently in clinical trials [4]. However, micro-dystrophins do not contain all of the functional elements and may have limitations compared to full-length dystrophin [79].

The only option to transfer the entire *DMD* gene is the use of **mammalian artificial chromosomes (MACs)**, which – like normal chromosomes – are replicated and portioned to daughter cells at each cell division [80]. A human artificial chromosome (HAC) carrying the entire human *DMD* locus (DYS-HAC) was introduced into **mesoangioblasts** from *mdx* mice, and the modified mesangioblasts were then directly injected into the skeletal muscles of immune-deficient *mdx* mice [81], resulting in effective engraftment and generation of functional muscle fibers. In a subsequent study, a DYS-HAC was transferred into human DMD satellite cell-derived myoblasts and perivascular cell-derived mesoangioblasts [82], which retained their myogenic differentiation potential *in vitro* and their ability to engraft the muscle of immunodeficient mice.

Although not clinically applicable, the SCNT technology in pigs offers an elegant route for studying the therapeutic potential of the DYS-HAC transfer strategy in DMD. Using **microcell-mediated chromosome transfer (MMCT)**, a DYS-HAC carrying a fluorescent reporter (EGFP) was introduced into fibroblasts from a male *DMD*Δ52 fetus [6]. EGFP-positive cells were used for SCNT. In all four resulting cloned piglets, the presence of the DYS-HAC was demonstrated by EGFP fluorescence and by PCR analyses documenting the presence of the full-length human *DMD* gene. The retention rate of the DYS-HAC (proportion of EGFP-expressing cells) in peripheral blood leukocytes ranged between 29% and 55% at age 1 month. Compared to untreated DMD pigs, the four cloned DYS-HAC piglets showed a delay in clinical symptoms and a slightly increased lifespan that was associated with initially lower serum CK values. Histological analysis of skeletal muscle samples of the cloned DYS-HAC pigs indicated a mosaic pattern of EGFP fluorescence in the muscle fibers and also a mosaic staining pattern of human dystrophin. Western blot analysis revealed <1% of WT dystrophin levels. At the time of necropsy (days 48, 52, 54, and 89, respectively) the retention rate of the DYS-HAC in peripheral blood leukocytes and several organs was estimated to be 20–24% and 4–39%, respectively, with significant variation among different organs [6]. The resilience of MACs in transchromosomic animals might be influenced by the compatibility between the species donating the artificial chromosome and the host species. In transchromosomic mice, murine artificial chromosomes proved to be more stable than HACs [83,84].

Phenotypic alterations in DMD mutation carriers

Although females with heterozygous *DMD* mutations (DMD carriers) seldom display clinical symptoms, they may occasionally experience muscle weakness and heart failure [85]. The likelihood of cardiomyopathy in DMD carriers rises with age [86]. Consequently, it is recommended that adult DMD carriers undergo echocardiography every 5 years [87]. The distinctive features of carriers, encompassing the onset and locations of pathology, as well as the severity of symptoms, remain underexplored. A breeding colony of DMD pigs is an important resource for characterizing the spectrum of clinical and molecular alterations in $DMD^{+/-}$ carrier pigs with a defined *DMD* mutation in a standardized environment.

A total of 29 *DMD*$^{+/-}$ carrier pigs with the *DMD*Δ52 mutation [66,67] from three generations were investigated for a maximum of 32 months [9]. Five animals (17%) died shortly after birth or at 1, 2, 4, and 7 months of age, respectively (Figure 3A). Regular measurements of serum CK activity in *DMD*$^{+/-}$ pigs revealed a broad range of values from normal to highly elevated (Figure 3B), confirming previous observations [8]. On average, the DMD carrier pigs showed a 10-fold increase in serum CK levels [9], which corresponds to studies in human patients where 70% exhibited increased CK levels [88].

Immunohistochemical and histopathological analyses of skeletal muscle tissue from 12 asymptomatic *DMD*$^{+/-}$ carrier pigs at 1.5–32 months of age revealed a mosaic pattern of dystrophin staining in young animals, whereas the pattern was more uniform in older animals. Degeneration

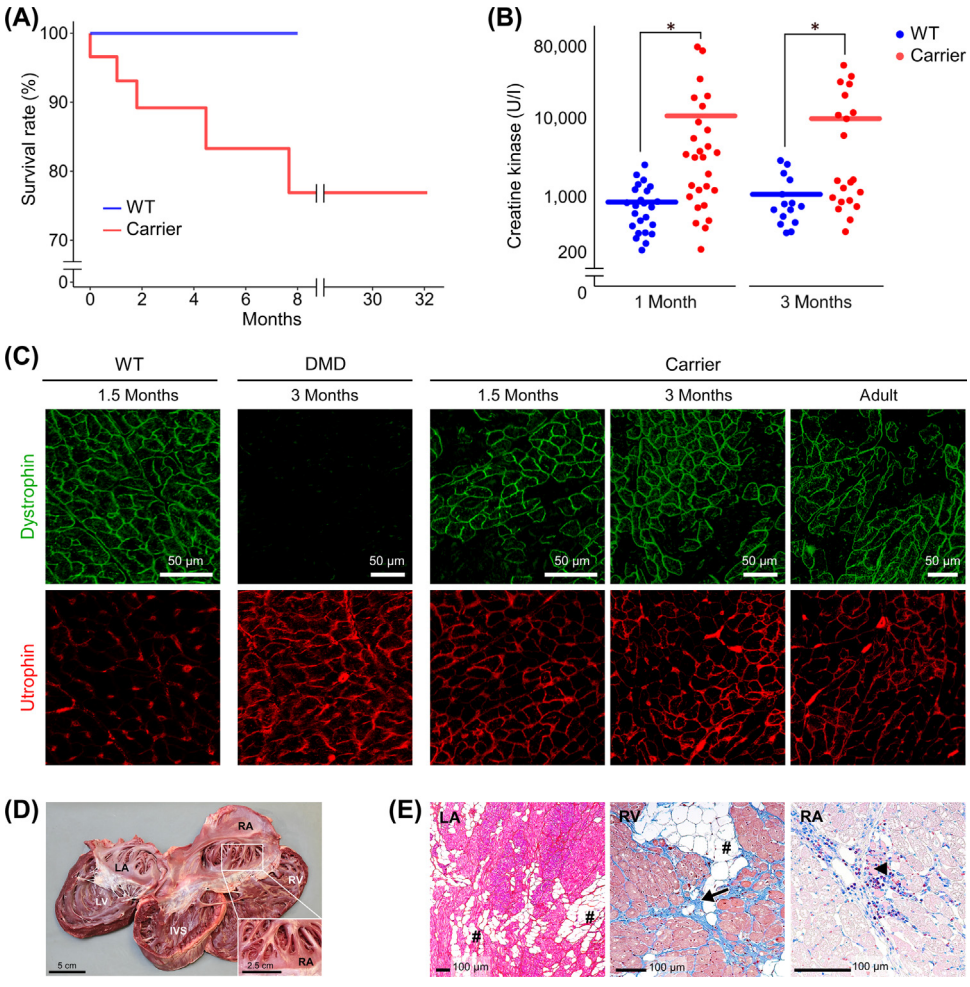

Figure 3. Phenotypic features of *DMD*$^{+/-}$ carrier pigs. (A) Survival rate; (B) serum creatine kinase (CK) activity; (C) dystrophin and utrophin immunofluorescence analysis of myocardium sections (panels A–C adapted, with permission, from [9]). (D) Macroscopic alterations of myocardium of a 7-year-old founder carrier sow [8]. Locations are indicated. Changes include severe dilation of the RV, patchy discoloration of the epicardium and myocardium, corresponding to replacement lipomatosis and fibrosis. (E) Histology documenting replacement lipomatosis (indicated by #), fibrosis [17], and multifocal myocyte degeneration with inflammatory cell infiltrates (arrowhead). Symbols and abbreviations: *, P <0.05; IVS, interventricular septum; LA, left atrium; LV, left ventricle; RA, right atrium; RV, right ventricle; WT, wild type; U, units.

of muscle fibers was observed in three of the six young individuals, whereas skeletal muscle tissue of adult pigs exhibited no degeneration, and infiltration of immune cells was observed in only one of six individuals [9]. Dystrophin immunostaining of *DMD*$^{+/-}$ myocardium sections revealed a mosaic pattern of dystrophin-positive and -negative cells, with an overall reduced abundance of dystrophin compared to WT myocardium and an apparent compensatory upregulation of utrophin (Figure 3C). In older *DMD*$^{+/-}$ pigs, dystrophin-deficient regions of the myocardium displayed immune cell infiltration and fibrosis [9].

The 7-year-old *DMD*$^{+/-}$ founder sow of a colony with the *DMD*Δ52 mutation was clinically healthy throughout her life, but necropsy revealed severe dilation of the right ventricle of the heart associated with white, patchy discoloration of the epicardium extending into the myocardium (Figure 3D) [8]. Histology showed multifocal myocyte degeneration with inflammatory cell infiltrates and replacement lipomatosis and fibrosis of the myocardium (Figure 3E). Histopathological changes of skeletal muscle, such as increased fibrosis, muscle cell necrosis, T cell infiltration, and mosaic staining of dystrophin-positive and -negative fibers, as found in human patients [89], were also observed in individuals of the DMD carrier pig cohort.

These observations indicate that *DMD*$^{+/-}$ pigs resemble the whole spectrum of possible alterations in human DMD carriers. The phenotypes of human *DMD* mutation carriers can vary due to skewed patterns of X-chromosome inactivation [90,91]. A dedicated biobank from *DMD*$^{+/-}$ pigs including blood, urine, and tissue samples [8] will be useful for the discovery of biomarkers to predict the risk of a severe phenotype, thereby offering the possibility of early therapeutic intervention.

Application of DMD pig models for testing novel diagnostic tools

In addition to enabling testing of different therapeutic approaches, DMD pig models can be used to validate novel diagnostic tools. Currently, the 6 minute walk test remains a crucial parameter in diagnosing DMD and plays a significant role in clinical trials [92]. However, it relies on the motivation and walking ability of the patient, and poses a risk of falls if motivation outweighs muscle strength.

Muscle **magnetic resonance imaging (MRI)** has emerged as a reliable, noninvasive means of measuring dystrophic changes such as edema, fat infiltration, muscle volume, and tissue composition, notably fatty degeneration. A recent systematic review [93] highlighted strong correlations between MRI findings and motor function. However, its routine use is hindered by lengthy scans, necessitating sedation for young or uncooperative patients, and restricting its practical application.

Multispectral optoacoustic tomography (MSOT) is an imaging technique that enables the precise *in vivo* detection of specific biomolecules within a tissue. This method combines optical illumination with ultrasound detection. It involves the use of a nanosecond pulsed laser in the near-IR spectrum to illuminate tissue, causing molecules to absorb light and convert it into heat. The sudden thermal expansion creates pressure waves within the tissue which propagate as acoustic waves that can be detected by an ultrasonic transducer and translated into an image [94]. Compared to light, these acoustic waves experience minimal scattering in tissue [95], allowing high-resolution contrast imaging even at considerable tissue depths. MSOT can differentiate between various tissue chromophores such as hemoglobin, melanin, lipids, collagen, and water by their characteristic absorption spectra, thereby enabling quantitative assessment of these molecules through spectral unmixing (Figure 4A).

A recent study [7] aimed to measure collagen levels in muscle tissue affected by progressive fibrosis in DMD using MSOT. After defining the parameters for collagen detection with MSOT, a longitudinal study on the biceps femoris and triceps brachii muscles of DMD piglets and age-matched WT

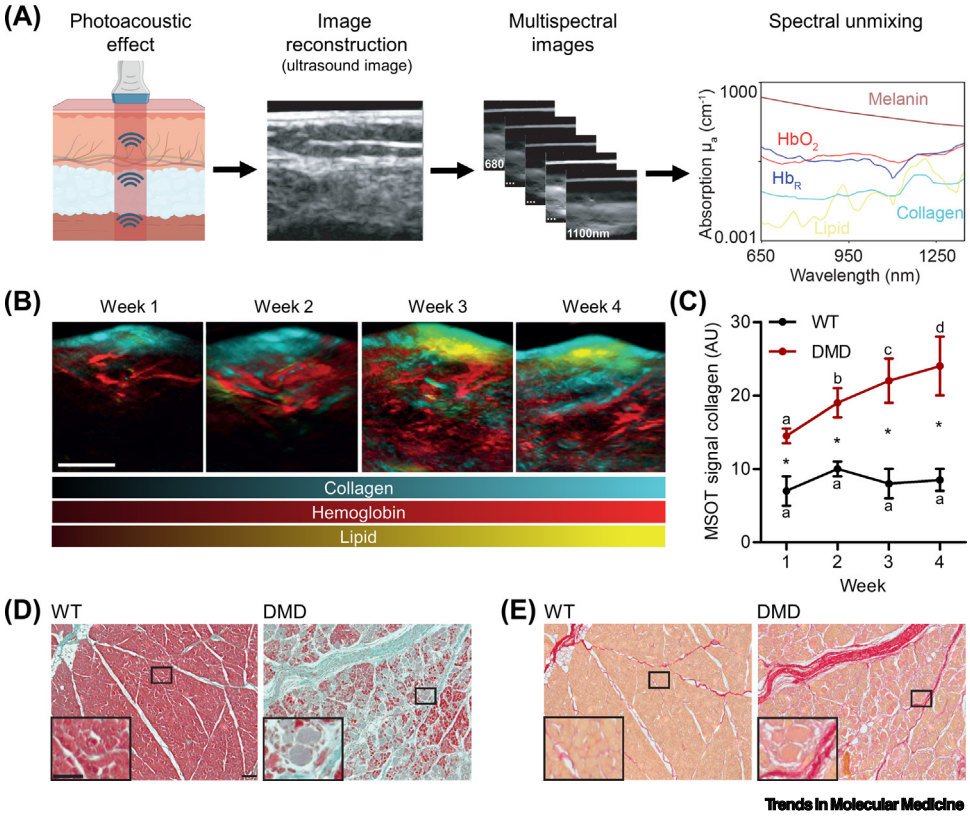

Figure 4. Longitudinal monitoring of muscle fibrosis in Duchenne muscular dystrophy (DMD) pigs using multispectral optoacoustic tomography (MSOT). (A) Principle of MSOT. MSOT combines laser-induced ultrasound and optical imaging to visualize tissues and structures within the body. The process begins with the illumination of the tissue using laser pulses. These laser pulses are usually in the near-IR spectrum, allowing a penetration depth into tissues of several centimeters while minimizing the compromise of resolution. When the laser light interacts with tissue, it is absorbed by endogenous chromophores (e.g., melanin, oxygenated and nonoxygenated hemoglobin, fat, collagen). The resulting thermoelastic contractions generate ultrasound waves (photoacoustic effect) which are detected by ultrasound transducers and used to reconstruct tomographic images of the internal structures. Data acquisition at multiple wavelengths allows the differentiation and quantification of various chromophores and enhances the imaging specificity. Thus, MSOT provides both anatomical and functional information such as oxygen saturation levels and collagen content at high resolution. (B) Representative muscle MSOT images of a DMD piglet over 4 weeks. (C) Quantification of 2D MSOT collagen signals in WT and DMD piglet muscles over time. Differences between groups at the same age are indicated by asterisks. Significant differences (P <0.05) between different time points within group are indicated by different letters. (D,E) Representative muscle histology of 4-week-old piglets after Masson trichrome (D) and Sirius red (E) staining. Scale bars: 100 μm and 50 μm (inset). Panels A–E modified, with permission, from [7]. Symbols and abbreviations: *, P <0.05; AU, arbitrary units; HbO_2, oxyhemoglobin; HBr, reduced hemoglobin; WT, wild type.

controls was conducted. While collagen signals remained stable in WT piglets, DMD piglet muscles showed a consistent increase in MSOT-derived collagen signals (Figure 4B,C). These findings were supported by histological staining (Figure 4D,E), hydroxyproline quantification, and specific collagen detection via mass spectrometry, confirming a quantitative relationship between MSOT-derived collagen signals and actual muscle fibrosis [7].

In an initial pediatric trial using MSOT, 10 DMD patients were compared to 10 age- and gender-matched healthy volunteers. This study revealed increased signals in various muscle regions of DMD patients, similar to each other but distinct from healthy volunteers. In addition, noteworthy negative correlations were found between MSOT-derived collagen signals and conventional

Clinician's corner

Genetically engineered pigs provide a unique opportunity to study monogenic human diseases in large animal models. The physiology of pigs is significantly more comparable to that of human patients compared to rodent models. In addition, ethical and moral concerns are less pronounced compared to non-human primates.

Specifically, the porcine DMD model has already been utilized to establish a new diagnostic tool for monitoring the progression of DMD patients [7]. Moreover, it has been used to evaluate the therapeutic potential of a gene editing trial in an animal model that closely mimics the body dimensions of humans [5].

The possibilities of gene editing, combined with well-established protocols for somatic cell nuclear transfer (SCNT), now enable the recreation of nearly every monogenic human disease in a porcine model, a practice that has been common in murine models for years. The higher costs of maintenance and increased generation intervals are more than compensated for by the greater comparability achieved with these porcine models.

clinical tests for tracking DMD progression. This established the potential of MSOT in the extended near-IR range as a clinically valuable method for non-invasively detecting collagens and monitoring disease progression in DMD [7].

Concluding remarks and future perspectives

As exemplified by the DMD pig, porcine models of monogenic diseases might help to bridge the gap between proof-of-concept studies in cellular or rodent models and clinical trials.

The availability of porcine models of DMD significantly broadens the range of organisms for translational DMD research. Given the ability to propagate DMD pigs through breeding, these models are accessible in substantial numbers for preclinical studies. In addition, the swift progression of the disease, especially the early manifestation of cardiac symptoms, allows the assessment of therapeutic efficacy within reasonable time frames. Therapeutic approaches demonstrating positive outcomes in preclinical trials using the clinically severe DMD pig model should also be effective in DMD patients, although this remains to be demonstrated.

Additional questions to be addressed in DMD pigs are alternative gene editing strategies, such as the deletion of *DMD* exons 45–55, that could be used for the majority of DMD patients [96]. By analogy to the *DMD*Δ51–52 pig model, *DMD*Δ45–55 pigs could be generated to predict the long-term outcomes of this strategy (see Outstanding questions).

It remains to be clarified in what way the restored dystrophin expression through exon-skipping therapy leads to autoimmunity against the expressed dystrophin in the pig model, similar to what has been described in humans [97].

In addition, the effects of modifier alleles (*trans*-acting genetic polymorphisms) affecting DMD disease progression [11] can be addressed in a targeted manner. Moreover, *cis*-acting genetic polymorphisms and rare variants [11] can also be explored in the pig model platform. The respective polymorphism can be engineered into *DMD* mutant pigs and – owing to their rapid progression of severe muscular dystrophy – the effects of the respective modifiers should be detectable within a reasonable time period. A recent example of modifiers are variants of the Tctex1 domain-containing 1 (*TCTEX1D1*) gene [98].

Finally, pigs can also be engineered to study physiological (and pathological) human muscle development following the so-called **exogenesis** approach. This concept is based on the formation of interspecies chimeras from pig embryos genetically engineered to lack a specific cell lineage and human stem cells that eventually form the missing lineage [99]. Recently, porcine embryos (up to day 27 of development) exhibiting a human myogenic lineage were successfully created [100]. This was achieved through complementation of pig blastocysts lacking **myogenic factor 5/6 (MYF5/6) and myogenic differentiation (MYOD/MYOD1)** with human **induced pluripotent stem cells (iPSCs)** that lack **tumor protein P53 (TP53)**. In principle the same pipeline could be used for human iPSCs carrying muscle disease-related mutations (such as *DMD*) to study their effects during early development.

The implementation of genetically tailored pig models can be extended to many other disease areas, thus promoting the translation of novel therapies and diagnostic procedures to clinical applications.

Outstanding questions

Will gene editing strategies to remove larger parts of the *DMD* gene, thus being applicable to a larger proportion of patients, be efficacious and what will be the long-term outcomes?

Will DMD pigs develop anti-dystrophin immune responses after therapeutic restoration of dystrophin?

How can delivery systems for gene therapy vectors or gene editing tools be optimized in terms of efficiency and tissue specificity?

What level of dystrophin is necessary to achieve a clinical benefit?

What are the mechanisms of *cis*- and *trans*-acting DNA polymorphisms in a model of clinically severe DMD?

Acknowledgments

Our projects were funded by the Bayerische Forschungsstiftung (AZ 802-08; PDOC-90-15), the Else Kröner-Fresenius-Stiftung (EKFS; 2015_180), the ForTra gGmbH für Forschungstransfer der EKFS (2018_T20; 2022_EKSE.41), the Meiji

University International Institute for Bio-Resource Research (MUIIBR), and the Japan Agency for Medical Research and Development (AMED; grant JP19gm0010002), and in part by the European Research Council (ERC; 101021043) and the Leducq Foundation (Network 23CVD01).

Author contributions

M.S., N.K., and E.W. wrote the initial draft of the manuscript. All authors revised the manuscript and approved the final version.

Declaration of interests

H.N. is a founder and shareholder of PorMedTec Co. E.W. is a founder and shareholder of MWM Biomodels GmbH and of XTransplant GmbH. These associations do not alter the adherence of the authors to journal policies on sharing data and materials. The other authors declare no conflicts of interests.

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