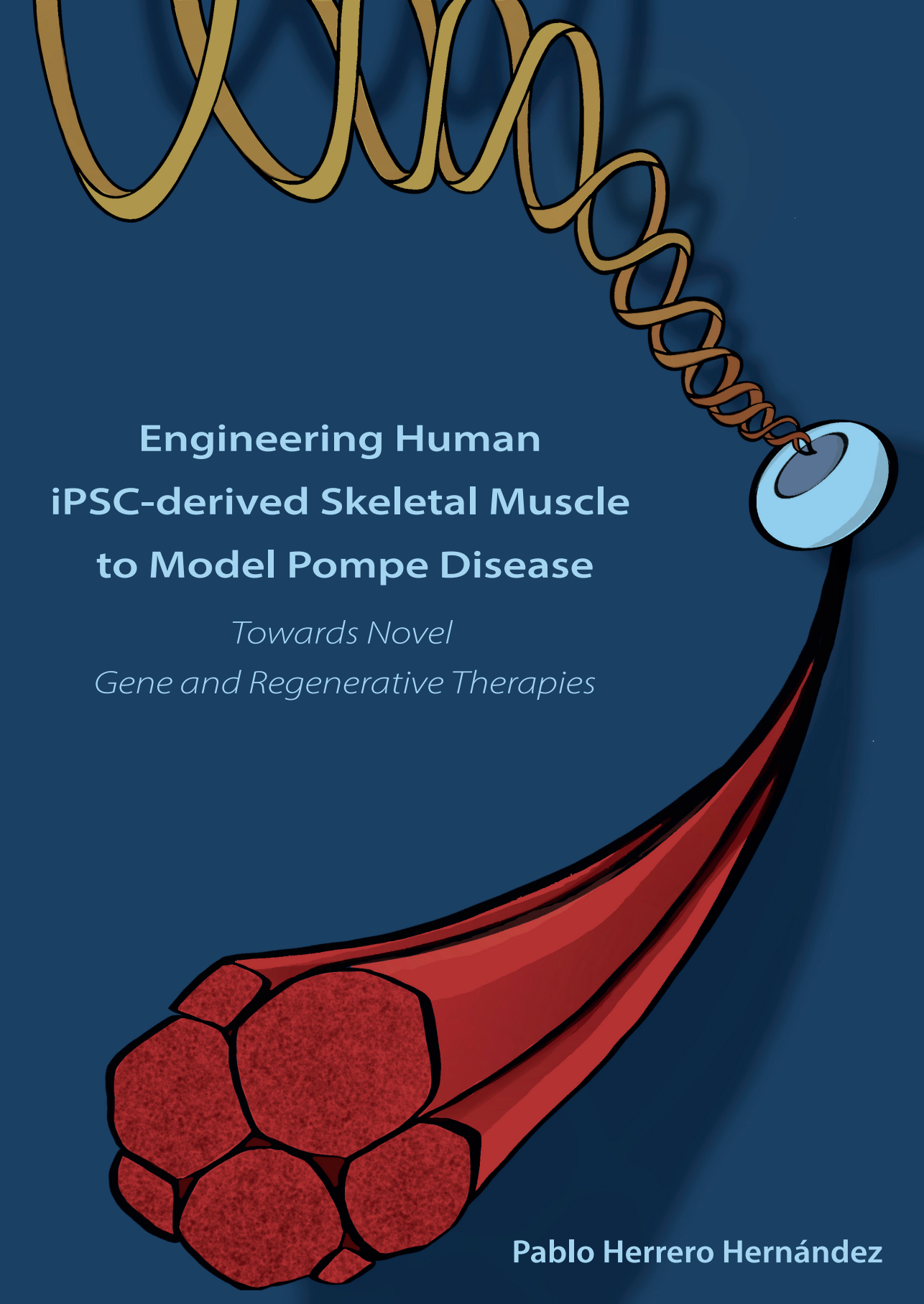

Engineering Human iPSC-derived Skeletal Muscle to Model Pompe Disease

Towards Novel Gene and Regenerative Therapies

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Engineering Human iPSC-derived Skeletal Muscle to Model Pompe Disease

Towards novel gene and regenerative therapies

Het modelleren van de ziekte van Pompe met behulp van spiercellen ontwikkeld uit geïnduceerde pluripotente stamcellen

In de richting van nieuwe gen en regeneratieve therapieën

Thesis

to obtain the degree of Doctor from the
Erasmus University Rotterdam
by command of the
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By

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A mis padres y hermana

To my parents and sister

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ABBREVIATIONS

4-MU	4-methylumbelliferyl-α-D-glucopyranoside
AAV	Adeno-associated virus
ACT	Adoptive cell therapy
ADA	Adenosine deaminase
ALL	Acute lymphoblastic leukemia
AON	Antisense oligonucleotide
ART	Antiretroviral therapy
CAR	Chimeric antigen receptor
cDNA	complementary DNA
CHO	Chinese hamster ovary
CK	Creatine kinase
CNS	Central nervous system
CRIM	Cross reactive immunologic material
CRISPR	Clustered regularly interspaced short palindromic repeats
DCs	Dendritic cells
DEGs	Differentially expressed genes
DMD	Duchenne muscular dystrophy
DSBs	Double strand breaks
ER	Endoplasmic reticulum
ERT	Enzyme replacement therapy
ESCs	Embryonic stem cells
FACS	Fluorescent activated cell sorting
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FSHD	Facioscapulohumeral muscular dystrophy
GAA	Acid α-glucosidase
GO	Gene ontology
GSD	Glycogen storage disease
GSEA	Gene set enrichment analysis
GvHD	Graft-versus-host disease
HbF	Fetal hemoglobin
HDR	Homologous end joining
HIV	Human immunodeficiency virus
HLA	Histocompatibility leukocyte antigen
HPV	Human papillomavirus

HSCs	Hematopoietic stem cells
IOPD	Infantile onset Pompe disease
iPSCs	induced pluripotent stem cells
IRAEs	Immune-related adverse effects
IVS1	c.-32-13T>G
KO	Knockout
LCA	Leber's congenital amaurosis
LOPD	Late onset Pompe disease
LSD	Lysosomal storage diseases
M6P	Mannose 6-phosphate
MEFs	Mouse embryonic feeders
MHC	Major histocompatibility complex
ML	Acute myeloid leukemia
MM	Multiple myeloma
MPCs	Muscle progenitor cells
MPS	Muchopolysaccharidosis
mRNA	messenger RNA
NGS	Next generation sequencing
NHEJ	Non-homologous end joining
NK	Natural killers
NMD	Neuromuscular disease
PAM	Protospacer adjacent motif
PD	Pompe disease
PMO	Phosphorodiamidate morpholino oligomer
pre-mRNA	precursor mRNA
QF	Quadriceps femoris
rhGAA	Recombinant human GAA
RNAi	RNA interference
RNA-seq	RNA sequencing
RNP	Ribonucleoprotein
RPE	Retinal pigment epithelium
RT-qPCR	Quantitative reverse transcription PCR
SCD	Sickle cell disease
SCs	Satellite cells
sgRNA	single guide RNA
SMA	Spinal muscular atrophy

SNVs	Single nucleotide variants
SSBs	Single strand breaks
TA	Tibialis anterior
TALEN	Transcription activator-like effector nucleases
tracRNA	Trans-activating CRISPR RNA
tTCR	transgenic T cell receptor
WT	Wild type
ZFN	Zinc finger nucleases

Chapter 1

INTRODUCTION

Introduction

Motion is described by physicists as the change in the position of an object over time. From subatomic particles to living matter like cells, motion is present everywhere. The movement of the human body arises from the interaction of internal forces generated within the body and external forces generated in the environment, like gravity. Voluntary movement in human beings is the result of contraction and relaxation forces conducted by one of largest tissues in our body, the skeletal muscle. The contractile force of individual muscle fibers is transmitted to the tendons, then to the bones, and is eventually translated into movement, posture and balance. Such contractile forces cannot not be accomplished without the help of other cell types. Skeletal muscle contraction occurs when motor neurons transmit a chemo-electrical stimulus from the nervous system to skeletal muscle fibers. When any of these components is affected by a genetic or non-genetic defect, muscle function may be compromised and movement is disturbed.

There are more than 1000 monogenetic neuromuscular disorders described so far[1] (Updated Dec 2020). Only few of them have a treatment option available so far. This is the case for Pompe disease, whose treatment was approved by regulatory medical agencies in 2006. With the advent of new gene editing, and gene and cell therapy technologies, current research is devoted to develop novel therapies based on single permanent interventions. However, concerns about unintended detrimental effects have paused its translation into the clinic. This has prompted researchers to develop strategies to minimize unfavorable outcomes including novel cellular models to test more accurately efficacy and safety. The ability of human induced pluripotent stem cells (iPSCs) to generate cell derivatives from the three germ layers (ectoderm, endoderm, mesoderm)[2] has opened the venue to generate large amounts of target cell types for disease modelling and cell therapy. However, tissue-specific cells generated from iPSCs are often immature and do not fully recapitulate the physiological properties of its analogs *in vivo*[3–6]. To understand the pathophysiology of neuromuscular disorders, more advanced models able to recapitulate the complexity of organs *in vitro* are required. *In vitro* tissue-engineered organs have greatly progressed to replicate key aspects of

organ function and could provide further understanding about drug mechanisms, disease pathology and stem cell biology. This introduction illustrates how precision gene editing, iPSC technology and skeletal muscle engineering can increase our understanding on human neuromuscular disorders and provide novel therapies, using Pompe disease as an example.

POMPE DISEASE

Pompe disease, also known as glycogen storage disease type II and acid maltase deficiency (OMIM 232300) is an autosomal recessive disorder caused by low or completely absent activity of acid alpha-glucosidase (GAA) which is required to degrade lysosomal glycogen to glucose. In 1932, the Dutch pathologist Dr. J. C. Pompe described a case of a 7-month girl who suddenly died of cardiac failure with hypertrophic cardiomyopathy and muscle weakness. Dr. J. C. Pompe observed massive accumulation of vacuolar glycogen in almost every tissue[7]. In 1963 Dr. H. G. Hers demonstrated that accumulation of glycogen was caused by deficiency of the acid maltase enzyme also described as GAA[8]. Pompe disease is a rare autosomal recessive disorder with an estimated frequency of one in 40,000, divided by one in 138,000 for classic-infantile and one in 57,000 for childhood/adult onset forms in Dutch and African-American populations[9,10]. Laboratory methods to diagnose Pompe disease combine biochemical assays and genetic analyses[11]. One of the most robust methods to diagnose Pompe disease is the measurement of GAA activity in fibroblasts obtained by skin biopsy[12].

LYSOSOMAL FUNCTION

The GAA enzyme is synthesized in the rough endoplasmic reticulum, transported to the Golgi network and finally transferred to late endosomes and lysosomes. Lysosomes are membrane-enclosed organelles that contain multiple enzymes capable to break down biological macromolecules (lipids, nucleic acids, proteins and carbohydrates). Lysosomes function as the digestive system of the cell, serving to degrade material taken up from outside of the cell and recycle dysfunctional components of the cell through autophagocytosis[13]. This traditional view of the lysosomes however, has been greatly expanded in recent years.

Lysosomes have been seen to exert multiple other functions beyond cellular digestion in response to environmental cues. For instance, they are involved in processes like metabolic signaling, gene expression, inflammation or membrane repair. They interact with other cellular components like the endoplasmic reticulum, Golgi complex, mitochondria, plasma membrane, autophagosomes or endosomes to control cellular homeostasis[14]. Defects in lysosomal function have been implicated in the pathogenesis of metabolic, neurodegenerative and cancer disorders. Most lysosomal enzymes, including GAA, are targeted to the lysosomes by exposure of the mannose 6-phosphate (M6P) residues on carbohydrate side-chains that are in turn recognized by M6P receptors in the trans-Golgi network. M6P groups can be sequentially added to N-linked oligosaccharides, hence allowing more than one M6P residue to be attached in nascent proteins. After binding to the receptor, the GAA enzyme is encapsulated in clathrin-coated vesicles that bud off from the trans Golgi network and are transported to late endosomes. Due to the low pH conditions of endosomes, both receptor and ligand uncouple and release the enzyme. Finally, active GAA reaches the lysosomes by autophagocytosis where the glycogen of the autophagosome is degraded[15].

CLINICAL SPECTRUM AND DIAGNOSIS

The deficiency of GAA results in accumulation of glycogen in the lysosomal compartment of almost every tissue, but in muscle (skeletal and cardiac) the consequences are more prominent leading to a wide spectrum of clinical manifestations. The age of onset is variable and depends in part on the disease-associated variants in the *GAA* gene [16]. The case described by Dr. J. C. Pompe typically represents the classic-infantile phenotype, a severe form of Pompe disease manifested shortly after birth. Symptoms include hypertrophic cardiomyopathy, hypotonia, feeding difficulties and failure to thrive. Typical symptoms include a head lag and inability to sit and roll over due to muscle weakness. In the absence of treatment, these patients do not survive beyond one year of age, developing heart and respiratory failure as the main cause of death[17]. Other forms of Pompe disease include childhood/adult onset and are characterized by a less progressive disease course without cardiac involvement. The symptoms

can present at any age. While the classic-infantile form is associated with less than 1% of GAA activity of the average normal activity in fibroblasts, the enzymatic activity in fibroblasts derived from patients with the childhood/adult onset form range from 3 to 20%. Patients with childhood/adult onset Pompe disease display progressive muscle weakness leading to mobility problems and respiratory insufficiency mainly caused by weakness of the diaphragm. These patients may eventually become wheelchair- and ventilation-dependent. Survival is reduced predominantly due to respiratory failure[18].

GENETIC HETEROGENEITY

The *GAA* gene is located on chromosome 17q25.2-q25.3 and encodes a 3.6kb transcript composed of 20 exons and introns. In Pompe disease, both *GAA* alleles need to carry disease-associated variants to manifest the disease. To date, more than 900 variants in the *GAA* gene have been described[19,20] (Pompe center - www.pompevariantdatabase.nl - updated Nov 2019). Variants that completely abrogate GAA activity are usually related to very early onset of Pompe disease like the classic-infantile form. Other less severe variants allow for residual expression and activity of *GAA.* The clinical manifestation is usually less than the classic-infantile form and a heterogeneous disease severity. Some mutations occur more frequently such as c.-32-13T>G (IVS1) which is found in more than 50% of the children and 80-90% of the adults with Pompe disease in the Caucasian population, but is never found among patients with classic-infantile form [21]. Patients with childhood/adult onset have almost always one allele with a less severe mutation that allows for some residual GAA expression and activity. Even patients with the same disease-associated variants, like siblings, manifest a variable age of onset and disease progression, suggesting that both genetic and environmental factors have an impact on the course of Pompe disease[22]. Patients with the classic-infantile form have on each of their two *GAA* alleles a mutation that leads to complete loss of GAA activity[23].

ENZYME REPLACEMENT THERAPY

To date, the only treatment available for Pompe disease is enzyme replacement therapy (ERT) approved by the European Medicine Agency and US Food and Drug Administration in 2006. This treatment greatly improved the prognosis of patients with classic-infantile Pompe disease and delayed disease progression in patients with later onset. It is based on the intravenous administration of recombinant human GAA (rhGAA) protein which is able to reach the affected tissues following uptake by the cells via M6P receptor-mediated endocytosis[24]. The recombinant enzyme used was first produced in rabbit milk, while the current product is derived from Chinese Hamster Ovarian cells[25]. ERT reduced the incidence of cardiac hypertrophy and improved major motor milestones like sitting and walking in classic-infantile form Pompe patients[26]. Fatal disease outcome like cardiorespiratory failure was greatly diminished or delayed beyond 1 year of age. However, the effect of ERT is more variable in patients with childhood/adult onset, leading to a heterogeneity response with respect to efficacy[27–29]. Still, at a group level, ERT showed a significant beneficial effect on the distance walked, pulmonary function and survival[30]. It is known that early diagnosis and start of treatment lead to the best results[31–33], however, childhood/adult onset patients may demonstrate symptoms which are only diagnosed as Pompe disease at an advanced state of the disease, limiting the efficacy of ERT. The limited effect of ERT in these patients is partly explained by the inefficient uptake of the rhGAA by the skeletal muscle tissue[34]. The demonstration that M6P receptors were also present at the plasma membrane and could mediate extracellular uptake of glycoproteins has led to the production of rhGAA, a 110 KD precursor exposing M6P groups[25,30]. Due to the low abundance of M6P receptors on the extracellular membrane of skeletal muscle cells, the low content of M6P groups on rhGAA obtained by large scale production in CHO cells, leaves room for improvement of ERT[24]. To address this issue, researchers have developed modified versions of rhGAA with a higher content of M6P groups (neo-rhGAA), which increased the delivery of the enzyme to muscle tissues[35]. Another reason why ERT may be less effective is that some patients develop antibodies against rhGAA which may diminish the effectiveness of ERT[36–

[38]. The combination of inefficient uptake, potential antibody formation and side effects and high costs of treatment are encouraging researchers to find novel strategies to treat Pompe disease.

STEM CELL BIOLOGY OF SKELETAL MUSCLE

Stem cell therapies rely on the intrinsic capacities of tissue-specific stem cells to regenerate injured tissues/organs. Fusion of healthy muscle cells (genome-edited or from healthy donor) with damaged muscle fibers could potentially reverse the course of muscle-degenerative diseases while other therapies like ERT may only stop or delay the progression of the disease[39]. Healthy muscle stem cells could also provide a long-term alleviation to the progressive deterioration of the muscle, since they self-renew to generate new progeny that will either form new muscle or re-populate the stem cell niche[40]. Albeit quite some challenges ahead to use this approach as a treatment for patients Muscle stem cells were first identified by transmission electron microscopy at the periphery of muscle fibers in 1961 and designated as “satellite cells”[41]. Located between the sarcolemma and the basal lamina[42], upon muscle injury, SCs are rapidly activated and differentiate into myoblasts and then myocytes which eventually fuse with each other or with existing myofibers to repair the damaged muscle[43] (Box 1). The stages of differentiation are molecularly characterized by the up- or down-regulation of myogenic transcription factors that are widely used to determine the commitment state of muscle progenitor cells. The paired box protein 7 (Pax7) is used as canonical biomarker for SCs as it is specifically expressed in all quiescent and proliferating SCs of many species[44]. Upon activation, SCs co-express MyoD, a basic helix-loop-helix transcription factor critical for myogenic commitment and differentiation, whose expression becomes gradually reduced at later stages of differentiation[45] (Figure 1). It lasted until 2005 that the first evidence was obtained that SCs cannot only efficiently regenerate damaged muscle but also repopulate the stem cell pool[43]. This provided robust evidence that SCs are *bona fide* muscle stem cells. Until then, myoblasts, of limited self-renewal capacity, were considered the most suitable sources for cell therapy[46–48]. Stronger evidence regarding the stem cell potential of SCs was obtained in 2008, when successfully engrafted SCs

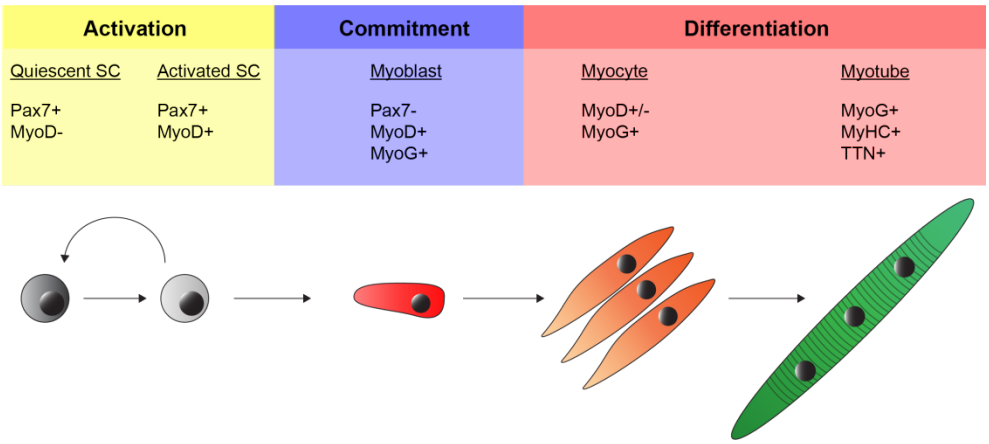

Figure 1. Stages of skeletal muscle differentiation from Satellite cells to myotubes. *SCs differentiate into committed muscle progenitors while maintaining a pool of muscle stem cells, ultimately differentiating further into multinucleated myotubes. For each stage, markers of skeletal muscle differentiation are shown.*

restored muscle function in dystrophic mice. This, opened the door for research on stem cell therapies for muscle disorders[49]. However, several studies also reported that freshly isolated SCs, although capable of engrafting and forming new muscle in mice, they lose their regenerative properties upon *ex vivo* expansion[50,51]. Due to the limited number of SCs within the skeletal muscle[40], expansion of muscle progenitor cells that retain their regenerative capacities becomes essential for the development of cell therapies for muscle diseases. Additionally, alternative sources of muscle cells with stem cell properties are needed rather than muscle biopsies from healthy donors.

DIFFERENTIATION OF HUMAN IPSCS TO SKELETAL MUSCLE CELLS

The recent development of strategies to efficiently derive skeletal muscle cells from pluripotent stem cells have emerged as a promising tool to improve our knowledge in skeletal muscle biology. Human iPSCs have the ability to generate many different cell types, including skeletal muscle[52]. This process can be replicated *in vitro* in two different ways: (1) Direct reprogramming methods are based on the overexpression of exogenous transcription regulators of skeletal muscle. The first

transgene-dependent myogenic conversion was achieved using MyoD in fibroblasts in 1987[53]. This breakthrough was followed by studies in embryonic stem (ES) cells and iPSCs using different transgene delivery vehicles[54–57] and was later followed by others using conditional overexpression of MyoD[58]. Similarly, Pax7, which is located upstream of MyoD in the myogenic lineage, has been used for the same purpose[59,60]. Direct reprogramming methods using transgenes are currently the most frequently used strategies to generate skeletal muscle cells from human iPSCs. This is partly explained by the robustness of these protocols which yield high numbers of myogenic cells after differentiation. These methods generate large amounts of skeletal muscle cells, however, they are based on the insertion of foreign nucleic acids which can lead to mutagenesis and alter the endogenous properties of muscle cells. (2) Directed differentiation procedures started in 2007[61], and use defined medium conditions to mimic the embryonic development of skeletal muscle *in vitro*. Independently of the conditions used, these procedures are divided in three major phases: 1) exit of pluripotency, 2) proliferation and 3) commitment. Vertebrate limb muscles arise from the paraxial mesoderm[62]. This early developmental step can be mimicked *in vitro* by activating WNT signaling in iPSCs, through inhibition of GSK3β[63–69]. Then, mesodermal progenitor specification and proliferation is successfully accomplished using growth factors involved in myogenesis like fibroblast growth factor 2 (FGF2). Finally, muscle commitment is induced by adding pro-myogenic factors like insulin, insulin-like growth factor or hepatocyte growth factor to the cultures. A significant improvement was recently obtained by using Bone morphogenic protein 4 (BMP4) ligand and GSK3β inhibition simultaneously[70,71]. Further studies reported an enhanced differentiation after concomitantly inhibiting transforming growth factor-β (TGF-β) signaling during the commitment phase[68,72]. Directed differentiation protocols generate skeletal muscle cells that are more similar to endogenous muscle cells present *in vivo*. However, these procedures result in large proportions of non-myogenic cell types that can influence subsequent experiments. Several recent methods combine direct reprogramming together with directed differentiation conditions[73,74]. These hybrid procedures accomplish efficient myogenic conversion of iPSCs using defined medium conditions with conditional forced transgene expression. First, the

exit from pluripotency and induction of proliferation of mesodermal progenitors is achieved, followed by conditional overexpression of myogenic factors to ultimately commit the expanding progenitors into the muscle lineage. Such combined approaches enabled to increase the yield of iPSC-derived muscle cell generation, yet, rely on transgene expression and still do not result in completely pure populations of muscle cells. To date, the only method to obtain pure muscle cultures derived from iPSCs is via fluorescent activated cell sorting (FACs). FACs-based protocols use markers expressed in muscle precursor cells like CMET, ERBB3, NGFR, CD10, NCAM and others, while excluding the non-myogenic neuronal fraction identified by HNK-1. FACs-purified muscle cells proved to form fibers *in vitro* and *in vivo* and represent suitable cellular models to study skeletal muscle biology in health and disease[61,63,65–69,74].

Despite significant improvement in recent years in the generation of transgene-free skeletal muscle cells from iPSCs, robust procedures able to increase the production yield of directed differentiation procedures will be crucial to achieve sufficient cell candidates for therapeutic implementation.

CELL THERAPY FOR NEUROMUSCULAR DISORDERS

Since the first evidence in 2008 that freshly isolated SCs could efficiently engraft and contribute to muscle regeneration in dystrophic muscles[49,51], limited progress has been achieved in developing efficient cell-based therapeutics for clinical use. Several limitations hamper the clinical use of SCs for cell therapy: 1) limited availability in the skeletal muscle. SCs can only be found in the skeletal muscle and it is estimated that 2-4 SCs are present per mm of fiber length in diverse muscles[75]. This illustrates the requirement for expansion of SCs *ex vivo*. 2) *Ex vivo*-expanded mouse or human SCs, or SC-derivatives, show an inefficient capacity to engraft[50,51,75–77]. Many attempts to increase numbers of regenerative muscle cells have been focused on optimizing *in vitro* conditions. For example, it is thought that culturing SCs in substrates that better mimic *in vivo* conditions could reduce the loss of engraftment potential. SC engraftment and expansion has also been improved through artificial substrates that replicate the SC niche. This has been achieved using bioengineered substrates, like soft hydrogels[78] or extracellular

BOX1: MUSCLE STEM CELLS IN POMPE DISEASE

SCs are *bona fide* muscle stem cells that are normally quiescent at the periphery of muscle fibers, but respond to skeletal muscle insults in order to restore tissue homeostasis. However, the regenerative capacity of SCs is often disrupted in muscle disorders. Pompe disease is caused by accumulation of glycogen in the lysosomes. The progressive accumulation of glycogen in skeletal muscle cells results in extensive vacuolization and defective autophagy[125]. These defects might eventually lead to impairment of sarcomeric contraction and possibly cell lysis. In such scenario, one would expect that the SC population would rapidly become activated to regenerate the injured muscle. While the SC pool remains unchanged in size in classic-infantile, childhood and adult onset Pompe patients, SCs in Pompe disease fail to become activated during disease progression[126]. The lack of muscle regeneration is not a result of compromised stem cell function, since they efficiently regenerated experimentally-induced muscle injury in different Pompe disease mouse models[127,128]. Further studies will need to elucidate the mechanisms behind the lack of SC activation and determine potential SC effectors that could improve the disease outcome in the skeletal muscle of patients with Pompe disease.

matrix (ECM) components[79–81] (laminins, collagens) that replicate the elasticity of the muscle tissue (12kPa). In addition, multiple small molecules involved in myogenesis or transcriptional regulation have been successfully tested. For example, inhibition of p38α, involved in SC commitment and highly expressed in aged SCs, could reduce their age-associated self-renewal defects[82]. Pharmacological manipulation of STAT3 (also elevated in aged SCs) could increase SC expansion and improved muscle repair in aged and dystrophic models[83]. A recent example of enhanced self-renewal and muscle regeneration was reported studying the pharmacological inhibition of Setd7, a methyltransferase

with limited known roles in the skeletal muscle[84]. Other studies have demonstrated that pro-inflammatory signals can benefit long-term SC expansion *ex vivo*. This is partly explained because skeletal muscle regeneration is completely abrogated without a balanced inflammatory response[85]. Inflammatory cytokines with known pro-myogenic roles involve IL-1α, IL-13, TNF-α and IFN-γ[86]. Additionally, Prostaglandin E2 has also been exploited to enhance muscle repair[87]. Extensive research has been conducted towards more efficient expansion strategies of muscle stem/progenitor cells for cell therapy, but little of these have been studied in other muscle models rather than SCs. Alternatively, iPSCs-derived from patient' fibroblasts, can be differentiated into expandable myogenic progenitor cells (MPCs) as described previously[67], and are amenable to gene editing. Mouse or human iPSC derived MPCs are amenable to engraft and regenerate muscle as studied in different models of muscle diseases[88], yet at lower efficiencies than freshly isolated SCs. Further optimization of cell engraftment and functional restoration will be crucial to advance in novel therapies based on cell extracts to treat the skeletal muscle.

GENE EDITING IN NEUROMUSCULAR DISORDERS

The recent development of novel tools to generate precise genome alterations has opened numerous opportunities for multiple fields, from agriculture to biomedical sciences[89]. They include zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), Meganucleases (MNs), and clustered regularly interspaced short palindromic repeats (CRISPR) in combination with CRISPR-associated protein (CRISPR/Cas). In particular, the cost-effectiveness and versatility of the CRISPR/Cas platform is greatly enhancing biomedical research and is now being used in several clinical trials[90]. However, many aspects of these platforms need to be improved to address issues regarding safety, efficacy and delivery to bring precision medicine to the clinic[90]. In **Chapter 2** we discuss in detail the basics and versatility of the CRISPR/Cas platform and the challenges researchers are facing to bring precision gene-editing to the clinic. Currently, the first clinical trials using precision gene editing are ongoing to treat cancer and

hematological malignancies, infection diseases, eye disorders and lysosomal storage diseases[91]. We reviewed these clinical trials in detail in **Chapter 3**.

Genome engineering of neuromuscular disorders is also experiencing an increase in research strategies targeted to improve disease outcome[92,93]. Researchers have successfully applied multiple gene-editing approaches to correct disease-associated variants in Duchenne muscular dystrophy (DMD), myotonic dystrophy (DM), limb girdle muscular dystrophy (LGMD) and facioscapulohumeral muscular dystrophy (FSHD) among other myopathies[94]. The gene-editing approach is determined by the selected delivery strategy designed. Two different approaches can be distinguished: 1) *In vivo* gene editing administers the gene editing components directly into the individual with the aim to modify tissues/organs of interest. 2) *Ex vivo* gene editing isolates cells from patients and conducts the gene editing intervention "out of the body", in a controlled environment. Genome engineering platforms generate targeted genome breaks and use the cell's DNA repair mechanisms to introduce genomic changes of interest (Figure 2). Gene editing through homology-directed repair (HDR) has been used to repair disease-associated variants or insert foreign DNA elements in models of muscular disorders. HDR requires a donor template containing the desired sequence to replace, flanked by homology arms around the cut site. To correct DMD[95,96], LGMD[97] and DM[98] via HDR is however less efficient in non-proliferating cells like myofibers or quiescent SCs. Other more efficient gene-editing strategies exploit the capacity of cells to repair nuclease induced breaks via non-homologous end joining (NHEJ). This DNA repair mechanism is widely used by the cell to correct genomic cuts and results in the introduction of small insertions or deletions. This method has been used to alter pre-mRNA splicing of defective genes to enhance endogenous expression in DMD via exon-skipping[99,100] and in congenital muscular dystrophy (MDC) via intron deletion[101]. Others successfully removed repeat regions in myoblasts from DM1 and in mouse models[102]. Interestingly, some of these studies have been recently used to test efficacy and safety in canine models[103,104]. The CRISPR/Cas system is often used to induce cuts in the genome to conduct desired editing outcomes. The capabilities of the CRISPR/Cas system for genome editing have been extended beyond exploiting the cell's DNA repair mechanisms to

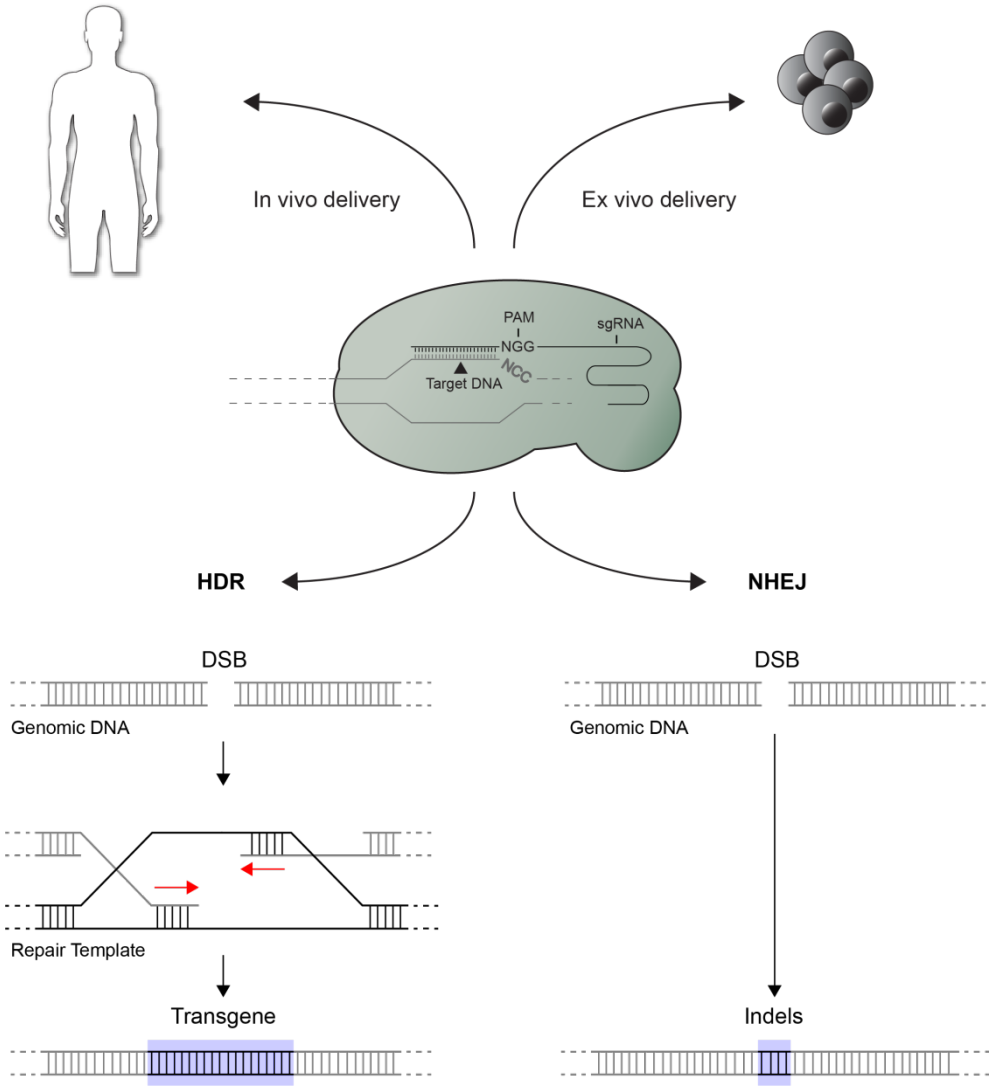

Figure 2. Gene-editing delivery strategies and DNA repair mechanisms used in (pre)clinical studies. *Natural and engineered CRISPR/Cas nucleases can be delivered directly into the individual to modify tissues and organs or "out of the body" in isolated cells which can be later transplanted into the individual. CRISPR/Cas nucleases exploit two major DNA repair mechanisms: Homology-directed repair (HDR) or Non-homologous end joining (NHEJ) to introduce genomic changes of interest.*

conduct genomic changes. Recent engineering approaches have created novel capacities to alter the genome. Base-editing uses an inactivated or dead Cas nuclease fused with a deaminase for C/G to T/A conversions or viceversa[105]. This strategy does not involve any cut on the genome therefore does not elicit a DNA

repair response and potential detrimental effects. This tool has been used to correct point mutations in the *Dmd* gene[106,107] among other disorders[105]. Alternatively, dead Cas nucleases have been fused with transcriptional activators or inactivators to ameliorate disease phenotypes in DMD[108], MDC[109], FSHD[110] and DM[111].

Despite the promising results achieved in multiple models for muscle disorders, the large size of skeletal muscle tissue as well as potential immune responses to gene editing and/or viral delivery vehicles have proven challenging. Precision genome medicine for neuromuscular disorders will need to attain sufficient safety and efficacy standards to prepare for entry into the clinic.

TISSUE-ENGINEERED SKELETAL MUSCLES FOR DISEASE MODELLING

With the development of iPSC and genome engineering technology, biomedical research has increased our understanding of human disease. This has traditionally been conducted using 2-dimensional (2D) cell culture and animal models. However, 2D cell culture systems lack sufficient cellular maturation and physiological complexity as observed *in vivo*. Animal models, on the other hand, do not completely recapitulate human diseases and drug responses. These limiting factors have prompted researchers to develop novel cellular models to better replicate human organ functions *in vitro*. Engineered tissues or organs-on-a-chip are cellular models that pretend to mimic the structural complexity and physiological properties of adult tissues using advanced 3 dimensional (3D) molds. These molds have to provide the appropriate structural organization and allow normal function of the desired tissue to study. Skeletal muscle is characterized by contraction and relaxation forces that allow movement. These are sustained by tendons attached to bones. Hence, engineered skeletal muscle models have followed a design where cells are attached to two structures, like pillars, made of a biocompatible, flexible material. Multiple designs of different size and shape exist to date, but all exploit the same principle to sustain and measure muscle contraction. Most engineered skeletal muscle models depend on scaffolds where cells are

embedded and cultured to mimic the native extracellular matrix of skeletal muscles[112]. Tissue-engineered skeletal muscles have been successfully used to measure contraction force and provided further cellular maturation in comparison to classical skeletal muscle culture models[113–115]. More recently, bioengineered skeletal muscle models have been created using human material. Generated using primary myoblasts[116,117] or muscle progenitors derived from iPSCs[118,119] engineered muscles have been used to model basic aspects of human muscle biology in health and disease, and to monitor drug responses. In a further attempt to increase the complexity of engineered muscle models and better mimic muscle physiology, different relevant cell types present in skeletal muscle were combined in a single 3D model. As such, researchers have successfully created vascularized skeletal muscle models *in vitro*[120,121] to increase the size of engineered muscles (thus allowing a better distribution of nutrients in the core) and model fibrosis in muscle dystrophies. Innervated muscle models with motor neurons[122,123] were generated to allow studying the neuromuscular junction and model drug responses in amyotrophic lateral sclerosis (ALS). Others have combined macrophages with engineered muscles to improve muscle regeneration *in vitro*[124]. These efforts have opened novel opportunities to conduct physiologically relevant experimentation in the laboratory that will greatly enhance our understanding of human skeletal muscle disease and will reduce the need for animal experimentation.

AIMS AND SCOPE

Aims

Significant achievements have been obtained using animal models to unravel fundamental aspects of skeletal muscle disease and to discover new drugs to improve muscle function. However, further drug development is hindered by our limited understanding regarding human disease pathology. Progress in efficient gene-editing tools and predictive *in vitro* models will greatly aid in the development of effective drug candidates for human disease treatment. The aim of this thesis is to explore the potential of iPSC technology, gene-editing strategies and tissue-engineering to better understand skeletal muscle wasting in Pompe disease. The strategies developed here could potentially be used for regenerative medicine and

to permanently correct disease-associated variants affecting Pompe disease individuals.

Scope

Precision genome engineering platforms, such as CRISPR/Cas9, are widely used for biomedical research. Targeted genome changes in iPSCs represent a promising tool for disease modeling and a potential source for regenerative therapy. In **Chapter 2**, we summarize the mechanisms of precision genome engineering tools to alter DNA/RNA, describe novel advances towards efficient gene-editing platforms and elaborate on safe delivery strategies for preclinical research. Multiple strategies exploiting CRISPR/Cas nucleases are being developed for the treatment of human disease. In **Chapter 3** we describe current clinical trials using CRISPR/Cas9 systems. Limited progress has been achieved towards safe delivery of gene-editing components directly to tissues/organs. Albeit most trials so far are intended to correct aberrant genome variants *ex vivo* for blood disorders, cancer and infection diseases, some progress to treat metabolic diseases is underway. In **Chapter 4** we describe a transgene-free method for the generation of pure, expandable skeletal muscle cells from iPSCs. Differentiating skeletal muscle progenitor cells are able to contract spontaneously *in vitro* and contribute to regenerate new fibers *in vivo*. Early drug discovery for skeletal muscle diseases is primarily done using primary muscle cells obtained from biopsies. These are difficult to obtain and demonstrate low expansion capacity in culture for high-throughput drug screening. In **Chapter 5** we elaborate a detailed description on how to identify novel AONs to restore the endogenous expression of altered genes using iPSC-derived skeletal muscle cells with Pompe disease as an example. In **Chapter 6** we describe two different gene-editing strategies to restore GAA activity in skeletal muscle cells derived from classic-infantile and late onset Pompe disease iPSCs. We use these genetically matched controls to investigate subtle changes at the transcriptome level and identify large gene expression differences between both Pompe disease forms. Further analyses determine district glucose utilization and muscle function defects linked to GAA activity levels. In **Chapter 7** we investigated how novel *in vitro* 3D skeletal muscle models could

be used to enhance our understanding regarding human skeletal muscle biology. We applied different types of injury commonly used in mice to study muscle regeneration and cell transplantation. The tools and procedures describe here could be used in the future to accelerate the development of regenerative-based therapies to restore skeletal muscle function.

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Chapter 2

Sharpening the Molecular Scissors: Advances in Gene-Editing Technology

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Review

Sharpening the Molecular Scissors: Advances in Gene-Editing Technology

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The ability to precisely modify human genes has been made possible by the development of tools such as meganucleases, zinc finger nucleases, TALENs, and CRISPR/Cas. These now make it possible to generate targeted deletions, insertions, gene knock outs, and point variants; to modulate gene expression by targeting transcription factors or epigenetic machineries to DNA; or to target and modify RNA. Endogenous repair mechanisms are used to make the modifications required in DNA; they include non-homologous end joining, homology-directed repair, homology-independent targeted integration, microhomology-mediated end joining, base-excision repair, and mismatch repair. Off-target effects can be monitored using *in silico* prediction and sequencing and minimized using Cas proteins with higher accuracy, such as high-fidelity Cas9, enhanced-specificity Cas9, and hyperaccurate Cas9. Alternatives to Cas9 have been identified, including Cpf1, Cas12a, Cas12b, and smaller Cas9 orthologs such as CjCas9. Delivery of gene-editing components is performed *ex vivo* using standard techniques or *in vivo* using AAV, lipid nanoparticles, or cell-penetrating peptides. Clinical development of gene-editing technology is progressing in several fields, including immunotherapy in cancer treatment, antiviral therapy for HIV infection, and treatment of genetic disorders such as β-thalassemia, sickle cell disease, lysosomal storage disorders, and retinal dystrophy. Here we review these technological advances and the challenges to their clinical implementation.

INTRODUCTION

In recent years, various platforms for genetically engineering somatic and pluripotent stem cells have been developed. They include zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALENs), meganucleases (MNs), and clustered regularly interspaced short palindromic repeats (CRISPR) in combination with CRISPR-associated protein (CRISPR/Cas). From agriculture to biomedical science, these platforms are being explored in various fields and, more recently, in the first clinical trials (Barrangou and Doudna, 2016; Naldini, 2015; Rodriguez-Rodriguez et al., 2019; Zhan et al., 2019).

Several developments are taking place in parallel. First, technological improvements and variations on gene-editing strategies are being reported with unsurpassed speed. Second, many possible applications are being developed to address a wide variety of biomedical questions. Third, the first platforms for gene editing are entering the clinical testing stage. Here, we follow these themes to present an overview of these recent developments, focusing on the likely clinical implementation of gene-editing strategies as well as discussing recent technological advances, and aspects such as safety, efficacy, and delivery that are relevant to clinical implementation. We provide a short overview of the progress gene editing is making toward applications in the clinic.

Technological Advances

Basics of CRISPR/Cas

Genome editing depends on the ability to generate specific pre-designed alterations in the genome. Inducing double-strand breaks (DSBs), single-strand breaks (SSBs) (also termed "nicks"), or specific base changes result in the activation of endogenous repair mechanisms that can be used to alter the genome. MNs, ZFNs, and TALENs were the first tools for genome editing in mammalian cells. MNs are naturally occurring endonucleases and can be re-targeted to new sites. Gene editing with ZFNs and TALENS results from the fusion of the FokI nuclease domain to the DNA-binding modules of zinc finger proteins (in the case of ZFNs) or transcription activator-like effector proteins (TALEs) (in the case of TALENS). However, the design and construction of these gene-editing tools can be labor-intensive, and their efficiencies for performing gene-editing varies. The discovery of CRISPR/Cas as a new gene-editing platform introduced a fast, cheap, and relatively efficient genome-editing method that revolutionized genome engineering.

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The mechanism of CRISPR/Cas is based on its role in adaptive immunity in prokaryotes, in which short stretches of invading foreign nucleic acids, so-called protospacers, are incorporated into the CRISPR locus of the bacterial or archaeal genome. After acquisition, CRISPR RNA (crRNA) is generated from the protospacers at the CRISPR locus. This crRNA can bind to complementary foreign nucleic acid and directs the Cas protein to recognize invading sequences. A second RNA known as the trans-activating CRISPR RNA (tracrRNA) is transcribed from a genomic locus upstream of the CRISPR locus and forms a complex with the crRNA. The crRNA:tracrRNA complex associates with a Cas protein (the nuclease) and creates an active ribonucleoprotein (RNP) complex that targets foreign nucleic acids for degradation (Mojica and Montoliu, 2016; Maeder and Gersbach, 2016).

For genome editing, the tracrRNA and crRNA are fused into a single guide RNA (sgRNA), which binds complementarily to a DNA target and guides the Cas protein to the desired target site, creating a DSB (Jinek et al., 2012). The target sequence is based on the presence of a protospacer adjacent motif (PAM), which is an absolute prerequisite for Cas protein to induce a DSB. The first publications on CRISPR arrays date back as early as 1987 (Ishino et al., 1987), Cas genes were discovered in 2002 (Jansen et al., 2002), and CRISPR/Cas was shown to cleave bacteriophage and plasmid DNA *in vivo* at specific sites in 2010 (Garneau et al., 2010). However, it was not until 2012 that two groups (Gasiunas et al., 2012; Jinek et al., 2012) adapted this system into a gene-editing tool. A review by Fernández and colleagues provides an extensive history of genome editing tools (Fernandez et al., 2017).

Its accessibility and relatively low costs have brought CRISPR/Cas a large number of applications in research worldwide and have catalyzed further research on understanding its mechanism of action, improving its functional capacities, and extending its biomedical applications. The CRISPR/Cas9 system originally applied has downsides that include a lower specificity than other gene-editing tools and a relatively large cargo size that hampers delivery to cells via vectors with limited cargo-size capacity (Fernandez et al., 2017; Guha and Edgell, 2017; Guha et al., 2017; Gupta and Musunuru, 2014; Zych et al., 2018). However, as we discuss below, innovative research has produced many adaptations to the original system that enhance its versatility and improve properties such as specificity and efficacy (Zhang et al., 2016; Wu et al., 2018).

Versatility of CRISPR/Cas-Mediated Gene Editing

As nucleases merely induce breaks in the DNA, the introduction of specific alterations in the genetic code requires the exploitation of distinct DNA-repair mechanisms. Intelligent engineering of the original genome-editing tools has broadened the scope of this toolkit, making it possible to achieve a great number of applications. As a result, CRISPR/Cas can be applied to interfere at multiple steps of gene-expression processes and can target processes at genomic and transcriptomic levels. Approaches include gene knock-out, precise correction of disease-associated variants, insertion of a cDNA in a safe harbor (a location in the genome—such as the AAVS1 locus—where there is no risk of insertional mutagenesis) (van der Wal et al., 2018; Sadelain et al., 2011); and manipulation of gene-expression regulatory elements such as promoter activity (Baliou et al., 2018) or splicing (Bergsma et al., 2018; Smith et al., 2018).

Targeted Deletion and Gene Knock-Out. The DNA repair pathway that is most commonly used to create deletions and specific gene knock-outs is non-homologous end joining (NHEJ). NHEJ repairs blunt or incompatible double strands, either by direct ligation or through mediation by microhomology of 5-25 nucleotides that flank the DSB to facilitate end joining (Chang et al., 2017a). NHEJ represents the major double-strand break repair system in mammalian cells and is active throughout the cell cycle (Chang et al., 2017a; Ranjha et al., 2018). Repair can be precise, leaving the target site intact for recleaving by the Cas nuclease. However, NHEJ-mediated DNA repair is rather error prone, as it can introduce random insertions or deletions of base pairs (indels) that will destroy the target site. These errors can result in a frameshift that inactivates gene products through mRNA decay (Ranjha et al., 2018). This effect is exploited in gene editing to create targeted gene knock-outs. Examples of clinical applications include knocking out disease-promoting genes such as oncogenes or restoring a reading frame by interfering with splice sites such as in Duchenne muscular dystrophy (Amoasii et al., 2018; Tabebordbar et al., 2016; Nelson et al., 2016).

Targeted Gene Editing and Knock-In. Traditionally, the homology-directed repair (HDR) pathway is used to achieve a precise knock-in. HDR recognizes DSBs and utilizes a homologous template—which,

under physiological conditions, is the sister chromatid—to repair the defect (Ranjha et al., 2018). For precision gene editing, this pathway is exploited by introducing a donor template that is used in the repair of the DSB rather than the sister chromatid. This donor template has the desired alterations, which are flanked by 3′ and 5′ homology arms on both sites of the DSB. In this manner, it is possible to accurately correct a point variant (as in cystic fibrosis) (Schwank et al., 2013); a tandem repeat (as in Fragile X syndrome) (Xie et al., 2016); large inserts or deletions (as in Duchenne dystrophy) (Li et al., 2015); or other genetic defects.

Due to the use of a donor template, HDR is a very precise repair pathway that is less error-prone than NHEJ. However, HDR is less active in cells and therefore much less efficient in genome editing than NHEJ, which limits its clinical potential. To favor its activation over NHEJ induction, its efficiency would have to be increased. This has cast new light on research into the regulation of DNA repair pathways (Mateos-Gomez et al., 2017; Schimmel et al., 2017; Zelensky et al., 2017). Several strategies using small molecules to inhibit NHEJ have been followed with varying success (Chu et al., 2015; Maruyama et al., 2015; Song et al., 2016; Yu et al., 2015; Pinder et al., 2015). Recently, cold shock was found to increase HDR in cells *in vitro* (Guo et al., 2018a). As HDR is highly suppressed in the G1 phase and its activity is limited to the S and G2/M phases of the cell cycle, it is restricted to dividing cells (Ranjha et al., 2018), which, since many cells in the human body are non-dividing, greatly restricts the clinical potential of HDR-based gene editing. Recently, Orthwein et al. have shown reactivation of HDR in the G1 phase via the PALB2-BRCA1/CUL3/Keap1 pathway. Although this reactivation might allow therapeutic targeting of non-dividing cell types, the limited efficiency seen to date would indicate the need for further research to demonstrate that therapeutically relevant HDR levels can be achieved through G1 reactivation (Orthwein et al., 2015).

More recently, other pathways have been used to achieve targeted knock-in. Through a technique named homology-independent targeted integration (HITI), NHEJ can also be harnessed to create knock-ins (Suzuki and Izpisua Belmonte, 2018). After a donor vector containing the desired transgene flanked by a CRISPR target site has been introduced into the cell, the donor vector and the genomic target are cleaved by Cas9, generating blunt ends on both genomic target and donor vector. These blunt ends are utilized to induce NHEJ-mediated end-to-end ligation, allowing the integration of the donor sequence into the target location (Sawatsubashi et al., 2018). As the activity of NHEJ is higher than that of HDR, this approach can be more effective and can be used in non-dividing cell types, because NHEJ remains active in all phases of the cell cycle (Suzuki and Izpisua Belmonte, 2018).

Several disadvantages limit the clinical potential of NHEJ-mediated knock-in. First, the transgene is inserted in a random direction. Second, due to potential off-target effects of CRISPR/Cas9 system, the use of a donor template may give rise to nonspecific insertions of this template. Last, NHEJ can introduce random indels, possibly disrupting the target location. However, clever vector design and target site selection can minimize the number of non-specific insertions and insertions in the wrong orientation (Suzuki and Izpisua Belmonte, 2018; Sawatsubashi et al., 2018).

Besides the classical NHEJ and HDR pathways, another DNA repair pathway has emerged in the last two decades: microhomology-mediated end joining (MMEJ) (also known as alternative end joining (a-EJ)) (Frit et al., 2014). In MMEJ, microhomologies exist upstream and downstream of the DSB site on the two DNA strands. DSBs with microhomologies can result in annealing of the microhomologies and subsequent repair by MMEJ, which can result in short deletions (Kim et al., 2018b). This pathway has been used to integrate a gene of interest by using a precise integration into target chromosome (PITCh) system in cultured cells, zebrafish, silkworm, and frogs (Sakuma et al., 2016; Nakade et al., 2014; Hisano et al., 2015). With a reported knock-in efficiency that is 2.5 times higher than that of HR-assisted gene knock-in (Nakade et al., 2014), and with activity during all phases of the cell cycle (Taleei and Nikjoo, 2013; Truong et al., 2013), this pathway opens up the possibility of more efficiently targeted gene knock-in in different phases of the cell cycle. Further work will be necessary to demonstrate the applicability and the feasibility of MMEJ-mediated knock-ins for precision genome editing.

Base Editing in DNA. Incorrectly repaired DSBs and DSB at off-target sites are potentially pathogenic. Attempts to circumvent the generation of DSBs, and thereby to lower the risks associated with gene editing, have resulted in the development of the base-editing technique (Hess et al., 2017; Molla and Yang, 2019), which exploits the natural function of cytidine deaminases to convert cytidine to uridine in DNA. Eventually, the uridine is converted to thymidine by DNA duplication or via the DNA repair mechanisms

base excision repair (BER) or mismatch repair (MMR) (Hess et al., 2017). Two different base-editing systems, BE (Komor et al., 2016) and target-AID (Nishida et al., 2016), have been developed by coupling cytidine deaminases to a catalytically deficient Cas9 protein (dCas9). Recently, a system with an incorporated adenosine deaminase was also developed. This enzyme hydrolyses adenosine into inosine, the base that pairs with cytidine. Inosine is thus replicated as guanine, thereby rendering an A to G change by DNA duplication (Gaudelli et al., 2017). These methods are clinically very interesting, as they open up opportunities for correcting monogenetic diseases in ways that reduce the risk of off-target effects. However, as most diseases are caused by various different disease-associated variants, this approach has to be tailored to each unique variant (Lessard et al., 2017). Another application of base editing is the introduction of a transcription-termination sequence to disrupt a gene in a highly specific manner (Kuscu et al., 2017; Billon et al., 2017).

Prime Editing. Recently, Anzalone et al. developed prime editing, a novel strategy for introducing deletions, inserting new genetic content or generating any of the 12 possible base-to-base conversions (Anzalone et al., 2019). For prime editing nicking Cas9 variant is fused to a reverse transcriptase, which, together with a prime editing extended guide RNA (pegRNA), makes gene editing possible. Prime editing offers a new range of possibilities in genome editing, with greater flexibility than the base editors, a greater reported efficiency than HDR, and no introduction of a DSB. Further research will determine the advantages and limitations of this promising novel concept.

Transient Modifications. All previous modifications result in permanent modifications at the genomic level. As an alternative approach, gene editing has been used to introduce transient modifications.

CRISPR interference (CRISPRi) was developed to inhibit gene transcription. Depending on the nature of the targeted locus, this approach is designed to interfere with transcription initiation or elongation (Bikard et al., 2013; Qi et al., 2013). The fusion of dCas9 to transcriptional activators or repressors has further sophisticated to the gene-editing toolkit for regulating transcription by CRISPR/Cas systems (Mahas et al., 2018).

The CRISPR/Cas system has also been engineered to modify epigenetic states by coupling dCas9 to several epigenetic modifiers such as P300 Core (Hilton et al., 2015), KRAB (Thakore et al., 2015), LSD1, Tet1, and Dnmt3 (Liao et al., 2017). Possible applications of this technique include the reversal of pathological epigenetic changes in conditions such as Fragile X syndrome, caused by silencing of the *FMR1* gene, which is associated with hypermethylation of the CGG expansion in the region encoding the *FMR1* 5′ UTR. Recently, Liu and colleagues showed that by using dCas9-Tet1 the CGG expansion can be demethylated, leading to reactivation of *FMR1* (Liu et al., 2018b). Future research is needed to test whether this is a valuable approach to treating fragile X syndrome and other epigenetic diseases.

Various studies over the years have shown that it is possible to target RNA with Cas13, an analogous member of the Cas family that is also referred to as C2c2 (O'Connell et al., 2014; East-Seletsky et al., 2016; Abudayyeh et al., 2016; Batra et al., 2017). One of these studies described the development of the RNA-editing platform known as RNA Editing for Programmable A to I Replacement (REPAIR) (Cox et al., 2017). For REPAIR a catalytically inactive Cas13 nuclease is coupled to a modified deaminase domain of adenosine deaminase that acts on RNA type 2 (ADAR2), which swaps A bases for I in RNA sequences. Additional modifications in the Cas13-ADAR2 fusion increased the targeted specificity of the REPAIR system almost a thousand-fold. In another study, protein engineering and characterization of different Cas13d orthologs generated a ribonuclease effector called CasRx (Konermann et al., 2018). The CasRx system has been shown to be highly effective in transcript knockdown or repression and in splice isoform manipulation.

Potentially, transcript editing could play a role in the therapy of diseases that cause transient changes in gene expression, such as local inflammation. As no definitive changes are made in the genome, but rather at transcriptional level, these approaches might represent a safer therapeutic strategy. However, the transient nature of these modifications will require repeated administration of the therapeutic agents.

Challenges in Bringing Genome Editing to the Clinic: Safety, Efficacy, and Delivery

Many conditions have to be met before genome-editing techniques can be considered for clinical development. Their efficiency and delivery must be great enough to attain a clinically significant result. Adverse events produced by permanent unintended variants should be minimized. Below, we highlight the

targeting specificities and efficacies of natural and engineered variants and the broad diversity of strategies for delivering gene-editing tools.

Specificity

Mechanisms of Undesired On-Target and Off-Target Effects. The development of precise gene-editing tools to treat genetic disorders in the clinic requires careful consideration of the medical implications of permanent modifications in the genome. Nucleases should provide sufficient targeted specificity to prevent potentially detrimental effects derived from DSBs and the subsequent DNA repair mechanism. These effects can include undesired small insertions and deletions, point variants, aberrant chromosomal rearrangements, and large deletions in edited cells (Kosicki and Bradley, 2018; Hsu et al., 2013); they occur at or around the targeted location ("on-target"), as well as at more distant locations in the genome ("off-target").

Influence of p53. p53 has been reported to cause a targeting selection bias in engineered cells: Cas9-gene targeting showed higher editing efficiency in cells with an altered p53 gene (Haapaniemi et al., 2018; Ihry et al., 2018), and transient p53 inhibition was shown to increase editing efficiency in human pluripotent stem cells, retinal pigment epithelial cells, and, more recently, in hematopoietic stem and progenitor cells (HSPCs) (Schiroli et al., 2019). However, due to the role of p53 in multiple DNA damage-response mechanisms, transient p53 inhibition may also leave cells more vulnerable to off-target mutagenesis. This idea has been challenged by others, who analyzed a large number of datasets derived from CRISPR screens (Brown et al., 2019; Ihry et al., 2019; Mair et al., 2019). These studies failed to show differences between p53-deficient and wild-type cells with respect to the enrichment of essential genes, thereby arguing against selection for clones that have a defective DNA damage-response pathway in CRISPR screens. Recently, guidelines were proposed for the performance of CRISPR screens with respect to monitoring and ensuring the quality of the screening performance (Brown et al., 2019).

R-Loop Formation. In addition to the risk of cleavage at an undesired location, the formation of R-loop-linked mutagenesis poses a risk. CRISPR/Cas cleavage is initiated by forming an R-loop, i.e., RNA-guided DNA unwinding to form an RNA-DNA hybrid with a displaced DNA strand inside the Cas protein (Jiang et al., 2016; Szczelkun et al., 2014). Although this R-loop is vital for stable binding and cleavage by the Cas protein, recent evidence in yeast indicates that the R-loop itself promotes mutagenesis on both on- and off-target sites (Laughery et al., 2019). This implies that undesirable mutagenesis may occur in many applications based on dCas9-fusions that were previously presumed to be safe, such as epigenome editing, transcriptional repression, and activation.

Effects on Transcription and Translation. Another risk is imposed by the introduction of indels via NHEJ, which can have unanticipated impacts on the regulation of RNA products and the translation of the protein it encodes. This includes promotion of internal ribosomal entry and alternative spliced mRNAs that lead to alternative products with a gain-of-function or a partially functional protein (Thomas et al., 2019; Mou et al., 2017; Tuladhar et al., 2019). mRNA decay can also trigger transcriptional adaptation. Via this genetic compensation mechanism, the expression of targeted or related genes can be upregulated independently or through protein feedback loops (El-Brolosy et al., 2019). Because the underlying mechanisms involved in splicing regulation and genetic compensation remain poorly understood, it remains a challenge to anticipate the translational and transcriptional changes induced by NHEJ-mediated indels.

Off-Target Effects of Base Editing. Because base editors do not produce DSBs in the genome, they do not pose risks of unintended damage triggered by DSBs. However, recent studies have reported the production of other types of off-target effects by adenine and cytosine base editors. In mouse embryos, variants using the APOBEC1 cytosine deaminase have been reported to generate multiple single-nucleotide variants (SNVs) at frequencies 20 times higher than the spontaneous mutation rate (Zuo et al., 2019). Other studies observed similar results, not only with the cytosine deaminases but also with the adenine editors based on the TadA deaminase, albeit at much lower frequencies (Kim et al., 2019). To reduce the rate of mutagenesis, Kim and colleagues coupled TadA with engineered Cas9 variants, successfully reducing their off-target activity (Kim et al., 2019). In addition, tens of thousands of SNVs were recently identified in RNA transcripts induced by both adenine and cytosine editors (Grunewald et al., 2019a; Zhou et al., 2019). These studies raised concerns about potentially detrimental variations not only in the genome but also in the transcriptome. Appropriate monitoring strategies will therefore be needed to evaluate the DNA and RNA of

engineered cells that are created with base editors. To enable the safe use of base editors in the clinic, further research on novel variants or on recently engineered deaminases (Grunewald et al., 2019b) may reduce their off-target effects.

Monitoring Undesired On-Target and Off-Target Effects. Off-target mutations are more difficult to detect than on-target variants, as they can be present anywhere in the genome. The most common workflow applied to date is to design gRNA sequences with minimal predicted off-target effects and perform targeted sequencing to those sites with a high predicted value. Many tools are publicly available, the choice depending on the model organism of interest (Listgarten et al., 2018). A recent comprehensive study by Allen and colleagues investigated the outcome of more than 40,000 Cas9-edited DNA sequences using different cell types (Allen et al., 2018), showing how different cell types preferentially use specific repair mechanisms for certain DNA sequences. This research led to the development of a prediction tool (FORECasT) that could be used to predict off-target effects.

Common monitoring methods for detecting on-target mutations are based on polymerase chain reaction (PCR) (reviewed in Zischewski et al. [2017]). Each assay is designed according to the mutation introduced into the host genome, and the method selected must detect the difference in the heteroduplex DNA in order to determine the editing outcome. Mismatch cleavage assays, such as T7E1 or Surveyor, are commonly used to identify indels in bulk and single-cell preparations. Although these methods are simple and effective in detecting small indels, they underrate the detection of large deletions or insertions. Additional methods that may provide detailed information about introduced mutations are based on Sanger sequencing.

Considering our limited knowledge of the mechanisms involved in CRISPR/Cas off-target activity, unbiased methods should be included that do not rely on predictions. Although whole-genome sequencing offers an unbiased high-throughput assessment of unintended variants, it is expensive and only provides information on bulk genomes. When applied to cell clones that have been edited *ex vivo*, whole-genome sequencing provides a valuable option. However, when applied to cell populations without clonal expansion, whole-genome sequencing is less suitable, as it may fail to detect low-abundant events that might eventually lead to oncogenic transformation. Other methods for detecting off-target effects are based on the fact that nucleases generate breaks that are repaired by endogenous DNA repair mechanisms. These have been exploited to develop techniques for quantifying the DSBs that have occurred *in vitro* (BLESS, GUIDE-seq, Digenome-seq) (Zischewski et al., 2017).

The selection of the most suitable detection method will depend on the sensitivity, throughput, limitations, and cost of the technique, and ultimately on the editing strategy (*ex vivo* or *in vivo*) and delivery system used (viral or non-viral). A recent study by Akcakaya and colleagues describes a robust evaluation of off-targets using *in vitro* assessment of potential off-targets via CIRCLE-seq, followed by *in vivo* targeted deep sequencing of engineered organs (VIVO) (Akcakaya et al., 2018). In a more recent study, Wienert and colleagues developed an unbiased monitoring strategy (DISCOVER-Seq) to evaluate potential off-target DSBs in cells and tissues (Wienert et al., 2019). Because these approaches are based on pre-repair mechanisms, additional detection methods will be required to evaluate their editing outcome and cytotoxic effect. In human diseases, robust standardized assessment guidelines will therefore be essential for precise genome engineering.

Increasing Specificity. Research has made great advances in the characterization of novel CRISPR/Cas9 orthologs and homologs from various species and in the generation of engineered enhanced-specificity Cas enzymes and sgRNAs. The most commonly used CRISPR/Cas system in research exploits the Cas9 protein derived from *streptococcus pyogenes*. However, many other orthologs offer different targeting abilities and specificities (Karvelis et al., 2017). Other natural homologs discovered recently include Cas12a (Cpf1) and Cas12b (C2c1), which can also be engineered for enhanced DNA specificity (Wu et al., 2018).

Recently, many engineered forms of Cas9 proteins with improved and broad targeting specificities have been developed. For example, Cas9 nickases (Ran et al., 2013) or Fok I fused to dead Cas9 nucleases (Tsai et al., 2014) use two different sgRNAs to perform ssDNA breaks, which can significantly reduce the off-target effects of WT Cas9 variants. Cas9 nickases have been used to generate paired single-strand DNA breaks in donor plasmids and at genomic target sites, thereby increasing gene-targeting efficiency,

specificity, and fidelity in human cells when compared with nicking targeting DNA alone (Chen et al., 2017b).

More recently, new engineered Cas9 proteins with enhanced targeted specificity have raised the possibility of using smart-designed nucleases for precise genome editing. The high-fidelity Cas9 (Cas9-HF1) (Kleinstiver et al., 2016) was designed by altering the composition of four residues involved in non-specific interactions of Cas9 with its target DNA. These modifications reduced the generation of mismatches with minimal loss of on-target efficiency. Using GUIDE-seq to monitor the frequency of off-target effects showed that editing with Cas9-HF1 resulted in only one single mismatch compared with the 65 detected for WT Cas9 using eight different sgRNAs toward four human genes in U2OS cells. The enhanced specificity Cas9 (eSpCas9) (Slaymaker et al., 2016) was created by modifying positively charged residues involved in the unwinding of the non-complementary DNA strand. Weakening this interaction reduced Cas9 off-target activity 10 times compared with that of WT Cas9, while maintaining on-target efficiency in human embryonic kidney cells. The evolved Cas9 (evoCas9) was generated through directed evolution in yeast (Casini et al., 2018), which involved the screening of random variants to identify beneficial variants in the REC3 domain involved in the recognition of the sgRNA and DNA heteroduplex. The combination of four beneficial variants generated the evoCas9 nuclease: a high-fidelity Cas9 variant with minimal loss of on-target activity and a reported 98.7% reduction in off-target activity relative to that in WT Cas9 when analyzed via GUIDE-seq (Casini et al., 2018).

The lack of mechanistic insight regarding target recognition of Cas9-HF1 and eSpCas9 led to the development of the hyper-accurate Cas9 (HypaCas9) (Chen et al., 2017a). It was shown that raising the energy requirements for the conformational activation of the HNH domain, which acts as a Cas9 editing checkpoint, reduced off-target activity in these variants. HypaCas9 was designed by modifying four amino acids involved in this process. Genome-wide specificity of this variant was significantly better than that of the WT Cas9 and equivalent to that of the Cas9-HF1 and eSpCas9 nucleases. On-target activity was at least >70% that of WT Cas9 when tested in U2OS cells.

Other Cas engineering strategies focused on increasing the targeting capacities of CRISPR/Cas nucleases by altering their PAM recognition sites without losing on-target specificity (Figure 1) (Kleinstiver et al., 2015; Hu et al., 2018). Directed evolution was used to create different variants of Cas9 nucleases with altered PAM targeting properties; the VQR and VRER variants demonstrated enhanced specificity in human cells. Other variants include xCas9, which recognizes a broader range of PAM sequences than natural Cas9. Recently, Nishimasu and colleagues designed a SpCas9-NG nuclease with a targeting capacity similar to that of the xCas9 variant. However, comparative studies showed that the editing efficiency of the SpCas9-NG variant was greater than that of xCas9. To convert C-to-T bases at NG PAM sites in human cells, the authors further combined SpCas9-NG with a cytidine deaminase, thereby providing new engineered Cas9 variants with many capacities beyond improved specificity (Nishimasu et al., 2018). Other homologs, such as Cas12a, have also been engineered to act on different PAM sequences instead of acting on their natural binding sites (Gao et al., 2017).

In addition, sgRNAs have been altered to enhance their targeting specificities. By modifying their length, secondary structure, or chemical composition, several studies demonstrated that off-target mutagenesis can be reduced (Fu et al., 2014; Hendel et al., 2015; Kocak et al., 2019). However, these changes may also influence their on-target efficiency. Subsequent studies found that partial replacement of RNA nucleotides with DNA could further reduce the off-target activity while retaining on-target efficiency (Yin et al., 2018). The optimal targeting specificity of sgRNAs will ultimately depend on the *in silico* design. Many software programs are publicly available for this purpose. However, it is important to consider the genetic variations between different individuals, as it can substantially alter the on-target and off-targeting activities of precise genome engineering (Lessard et al., 2017).

Spatiotemporal Control of CRISPR/Cas Activity. The capacity of nucleases to generate permanent genome changes has prompted researchers to seek novel methods of exerting spatiotemporal control over CRISPR/Cas activity. Such control is important: when targeting DNA to reduce undesirable off-target effects, any intervention in a desired tissue or organ should be brief.

The spatial localization of precision gene-editing tools can be manipulated by using different delivery vehicles. For instance, different serotypes of viral particles such as lentivirus or adeno-associated virus (AAV)

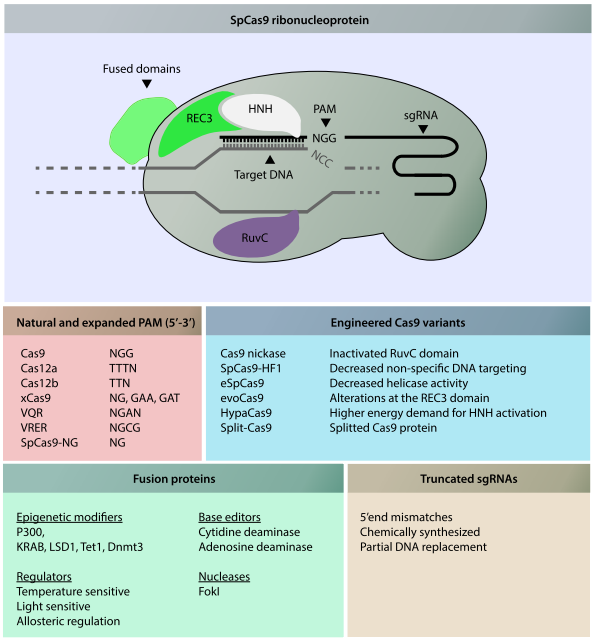

Figure 1. SpCas9 Ribonucleoprotein Variants with Altered Targeting Capacities

Engineered SpCas9 nuclease variants with potential therapeutic advantages. Variants with expanded PAM recognition sequences include natural Cas9 and its well-described homologs Cas12a and Cas12b. Designed engineered nucleases with improved specificity include the split-Cas9 variant, which was created to improve the packaging of Cas9 proteins in AAV vehicles. Cas9 nucleases have also been fused with multiple functional domains to allow for targeted epigenetic modifications, single nucleotide modifications, single-strand nicking activity, or temporal regulation of CRISPR/Cas activity. Truncated versions of sgRNAs have been successfully used to increase targeting specificity. SpCas9, streptococcus pyogenes Cas9.

have higher affinities for specific cell types. By using tissue-specific promoters, the expression of CRISPR/Cas components can be limited to target tissues or organs. Other organs can be targeted through local delivery with limited distribution into other tissues, such as the eye. However, for efficient targeting, diseases involving larger organs or tissues (such as muscle or skin) might require systemic administration.

Off-target effects are also magnified by extended gene-editing activity. Several approaches have therefore been developed to influence the spatiotemporal activity of CRISPR/Cas systems. Many natural Cas9 enzymes have been engineered into switchable Cas9 nucleases, either to be light sensitive, to be controlled by allosteric regulation, or to express specific localization signals (Richter et al., 2017). However, the clinical potential of these engineered forms can be limited, as some switchable nucleases respond to toxic regulators such as doxycyclin or UV light. Nevertheless, the characterization of novel natural inhibitors for Cas9 and Cas12a variants holds great promise for the therapeutic regulation of CRISPR/Cas activity in a clinical setting (Pawluk et al., 2016; Watters et al., 2018; Jiang et al., 2019; Uribe et al., 2019). Another approach that holds great potential for controlling the CRISPR/Cas system and off-target activity is the use of anti-CRISPRs. Discovered in phages to overcome CRISPR immunity, these anti-CRISPRs can be used, repurposed, and structurally engineered to control the CRISPR/Cas9 system (Pawluk et al., 2018).

Immunogenicity and Safety. Gene targeting must be performed with minimal toxicity and immunogenicity. The immunogenic properties of gene-editing components have recently been described in several studies (Charlesworth et al., 2019; Kim et al., 2018a; Wagner et al., 2019; Chew et al., 2016). CRISPR/Cas systems are protein complexes derived from bacteria and archaea, some of which, such as *S. aureus* (SaCas9) or *S. pyogenes* (SpCas9) are common infectious agents in the human population. The risks associated with immune responses against Cas9 proteins (or other Cas homologs) and sgRNAs should be considered in clinical trials. A recent study by Charlesworth and colleagues on the presence of preexisting antibodies reported that 78% of donors involved in the study presented antibodies against SaCas9 and 58% presented antibodies against SpCas9. In addition, analysis of blood samples indicated that 78% had anti-SaCas9 T cells and 67% had anti-SpCas9 T cells (Charlesworth et al., 2019). The detection of anti-Cas9 T cells might pose a serious challenge to precision genomic engineering, as exposure to a cytotoxic T cell response might eliminate engineered cells. Because the assays used in this study were not intended to investigate cellular immune responses against CRISPR/Cas components, this issue should be addressed in future studies.

Gene-editing tools used *in vivo* will be exposed to the host immune system. Potential immune reactions will be influenced by the type of delivery vehicle used. For instance, viral-based vectors could lead to long-term expression of gene-editing tools. These could lead to sustained nuclease activity, which, depending on the variant used, might in turn lead to extensive damage to the human genome and a prolonged immune response. In addition, immune responses to viral-based vectors such as AAV are known for their capacity to neutralize the therapy, including an induction of a cytotoxic T cell response that eliminates cells that have been targeted by the AAV vector (Mingozzi and High, 2011, 2013). This is highly relevant, as AAV is a naturally occurring virus against which ~40% or an even higher percentage of people already carry antibodies to AAV capsid proteins.

To minimize the effects of potential immune reactions, researchers can follow various strategies. In one approach, gene-editing activity can be spatiotemporally controlled, which has great potential for clinical use (see above for the section on *Spatiotemporal control of CRISPR/Cas activity*). Gene-editing components can for instance be directed to immune-privileged organs such as the eye or to tolerogenic organs such as the liver. Strategies involving immune modulation might prevent side effects derived from bacterial Cas proteins or from antigens derived from the delivery vehicles. An appropriate evaluation of candidate approaches and potential detrimental effects will be essential for the safe use of precision genome engineering in the clinic.

Delivery Strategies

The development of efficient and safe delivery systems is one of the most challenging aspects of introducing precise genome editing to the clinic. The delivery approach to be used will be influenced by the type of gene-editing material. So far, CRISPR/Cas systems have been successfully administered in naked or encapsulated plasmid DNA, mRNA, or functional (ribonucleo)protein complexes both *in vivo* and *ex vivo* (Figure 2).

In Vivo Delivery. In many clinical and pre-clinical studies, adeno-associated viruses are commonly used delivery vehicles *in vivo*. Different AAV serotypes provide increased delivery efficiencies for specific cell types, thereby allowing tissue/organ targeting (Colella et al., 2018). *In vivo* delivery of gene-editing machinery using AAV has been successfully used in multiple animal models of metabolic diseases (Pankowicz et al., 2016; Yin et al., 2016; Villiger et al., 2018; Rossidis et al., 2018), human immunodeficiency virus (HIV) infection (Yin et al., 2017a), muscle dystrophies (Amoasii et al., 2018), brain disease (Nishiyama et al., 2017), retinal disorders (Suzuki et al., 2016; Huang et al., 2017; Maeder et al., 2019), degenerative disorders (Beyret et al., 2019; Santiago-Fernandez et al., 2019), and diabetes and kidney malignancies (Liao et al., 2017). More recently, it has been used to upregulate the expression of endogenous genes in haploinsufficiencies such as obesity (Matharu et al., 2018).

Due to its high efficiency, viral delivery through AAVs offers promising results for precision genome-editing medicine. However, AAVs elicit immune responses that may limit the therapeutic potential of genome-engineering tools. If the gene-editing strategy should be administered repeatedly over time, this is especially relevant, as patients will develop antibodies against the AAV virus after the first administration, precluding any subsequent treatment using AAV as delivery vehicle. Furthermore, as a significant proportion of the

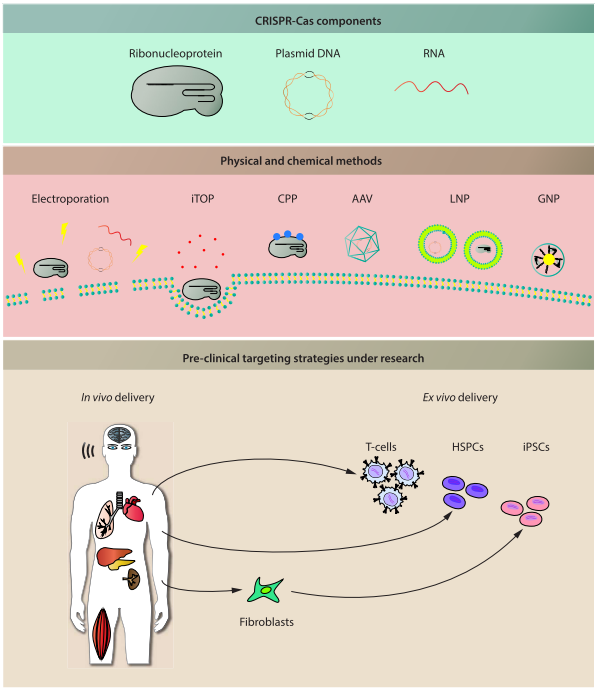

Figure 2. Delivery Strategies Used in Preclinical Studies

CRISPR/Cas gene-editing tools have been delivered as ribonucleoprotein complexes, plasmid DNA, or RNA. They can be delivered as naked components using chemical or physical methods or in delivery vehicles including virus and/or different types of nanocomplexes. There are two main strategies for delivering precision gene-editing platforms. In multiple organs and tissues in animal models, *in vivo* delivery strategies have been successfully used, some of which have been clinically approved for application in humans. *Ex vivo* delivery strategies are being extensively used in T cell engineering, hematopoietic stem-cell gene editing, and iPSCs modeling. iTOP, induced transduction by osmocytosis and propanebetaine; CPP, cell-penetrating peptide; LNP, lipid nanoparticle; GNP, gold nanoparticle, HSPCs, hematopoietic stem and progenitor cells; iPSCs, induced pluripotent stem cells.

population has pre-existing antibodies against the AAV virus, it is not eligible for AAV-based treatment (Louis Jeune et al., 2013). However, some of these limitations may be overcome by combined immunosuppressive therapies. AAV delivery can also drive long-term transgene expression and might result in low incidence of transgene integration. This promotes long-lasting gene editing in tissues and thus poses a high risk of off-target events. Methods for inactivating Cas activity would be required to prevent the long-lasting introduction of double-strand breaks *in vivo* and to reduce the risks of chromosomal abnormalities.

The large size of Cas proteins hampers their packaging into AAV plasmids. To circumvent this, researchers have successfully treated eye diseases using smaller Cas9 orthologs derived from *campylobacter jejuni* (CjCas9) (Kim et al., 2017). The study in question described how researchers were able to package the DNA sequence of the CjCas9, sgRNAs, and a donor template in a single AAV vector. Others have designed a flexible AAV-split-Cas9 platform to compact the Cas9 DNA sequence into AAV vectors (Chew et al., 2016). By manipulating the WT protein sequence of the Cas9 nuclease into fused functional domains, it was possible to shorten the newly engineered Cas9 DNA sequence by more than 2 kb.

Over recent years, different types of nanomaterial have been developed with encouraging results in multiple diseases (reviewed in Li et al. [2018]). Unlike AAVs, lipid-based nanoparticles are able to transfer Cas9 plasmids or proteins without the risk of genomic integration; some of them have received FDA approval for therapeutic use (Glass et al., 2018). In mice, lipid nanoparticles are currently used to deliver Cas9 components locally to the brain (Wang et al., 2016) or inner ear (Gao et al., 2018) and systemically to the liver (Yin et al., 2017b; Finn et al., 2018). However, for the delivery of Cas9 and a donor template for HDR, both components must be encapsulated in distinct lipid nanoparticles, which might affect its editing efficiency *in vivo*. More recently, newly developed gold nanoparticles have been successfully used in rodent models to simultaneously deliver Cas9 ribonucleoproteins and donor templates to treat Duchenne muscular dystrophy (Lee et al., 2017) and fragile X syndrome (Lee et al., 2018). Park and colleagues have shown successful genome editing of post-mitotic neurons in different Alzheimer disease mouse models using amphiphilic nanocomplexes generated using the R7L10 peptide together with the Cas9 nuclease and the sgRNAs (Park et al., 2019a).

Ex Vivo Delivery. Many vehicles are being exploited to deliver precision gene editing *ex vivo*. Depending on the type of cell or stem cell used for *ex vivo* editing, the gene-editing machinery may be delivered via electroporation, microinjection, chemical methods such as cell-penetrating peptides and nanoparticles (Wu et al., 2018), and virus-based vehicles such as AAVs or lentiviruses (Figure 2). The efficacy with which various types of immune cells can be engineered *ex vivo* has been investigated in multiple preclinical models, greatly stimulating research in several fields, particularly hematology and cancer therapeutics (Bak et al., 2018; Huang et al., 2018). Pluripotent stem cells such as induced pluripotent stem cells (iPSCs) have also been used extensively for genome engineering *ex vivo* for cell-based regenerative approaches and for disease modeling (Jang and Ye, 2016). iPSCs offer great potential for disease modeling, as they can be differentiated into any cell type that is relevant for the disease, such as cardiomyocytes (Brandao et al., 2017; Devalla and Passier, 2018), skeletal muscle cells (van der Wal et al., 2018; Magli and Perlingeiro, 2017; Chal and Pourquie, 2017), neuronal cells (Bordoni et al., 2018; Compagnucci et al., 2014), hepatocytes (Fiorotto et al., 2019; Hannoun et al., 2016), and many other cell types. Isogenic controls that correct for genetic background effects can be generated in iPSCs using CRISPR/Cas; this correction for background effects is considered important due to the large variation in many parameters among individuals. The risk of acquiring variants during the reprogramming of somatic cells into iPSCs has imposed the need for caution in the use of iPSC-derived cells for cell-based therapy. In addition, the development of affordable clinical treatment is inhibited by the cost associated with the quality control of patient-specific iPSCs. As a possible solution, the generation of iPS-cell banks covering the majority of HLA isotypes known globally is currently in progress. This would make a validated iPSC line available for cell-based therapy for almost each individual patient without the need to generate patient-specific iPSCs (Ben Jehuda et al., 2018; Mandai et al., 2017).

Preclinical Studies and Clinical Trials

Therapeutic gene editing by ZFN, TALEN, or CRISPR/Cas9 platforms is already being explored in a number of clinical trials registered at clinicaltrials.gov. As we discuss briefly below, these target cancer, genetic disorders, and HIV/AIDS (Figure 3).

Cancer

In HPV-related cervical cancer, CRISPR/CAS9, ZFNs, or TALENs are applied topically to precancerous lesions in order to disrupt viral oncogenes *E6* or *E7 in vivo* (Ding et al., 2014; Hu et al., 2014, 2015; Kennedy et al., 2014; Shankar et al., 2017; Zhen et al., 2014). In addition to induction of (viral) oncogene knock-out, gene editing for the treatment of various types of cancer is also being investigated in cellular immunotherapies or adoptive cell therapy (ACT). To overcome evasion of immune surveillance by cancer cells, *PD-1*, an immune checkpoint molecule, was knocked out *ex vivo* in autologous T cells using CRISPR/Cas9 before re-infusion into patients (Beane et al., 2015; Menger et al., 2016; Su et al., 2016; Zhao et al., 2018). *PD-1* knock-out have been deployed to improve redirected T cells (Guo et al., 2018b; Hu et al., 2019a, 2019b; Lu et al., 2019; Ouchi et al., 2018; Rupp et al., 2017; Su et al., 2017), which were genetically altered to express a transgenic T cell receptor (tTCR) or a chimeric antigen receptor (CAR) targeted at a disease-associated antigen. To enable ACT for T cell malignancies expressing CD7, CRISPR/Cas9 has also been used *ex vivo* to knock out the *CD7* gene in anti-CD7 CAR-T cells, preventing these CAR-T cells from killing each other (Cooper et al., 2018; Gomes-Silva et al., 2017). Finally, to circumvent the necessity for custom-made autologous therapy for each patient, *ex vivo* gene editing by CRISPR/CAS9 or TALENs in CAR-T cells manufactured from

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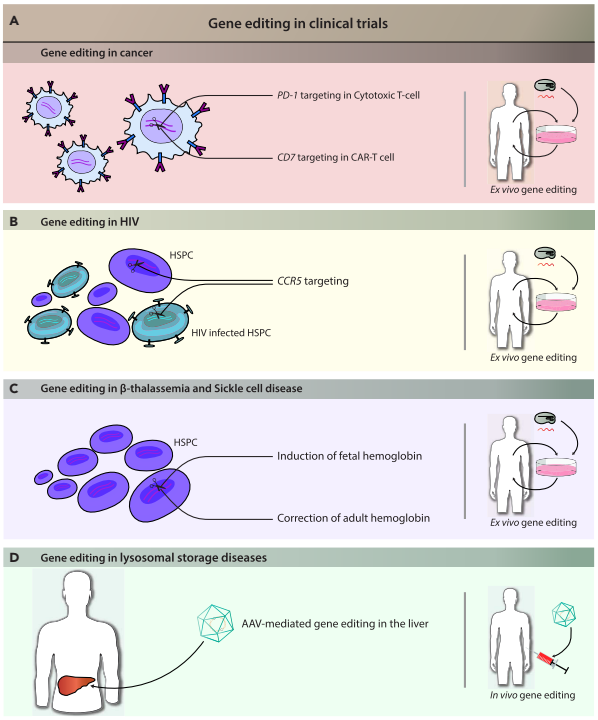

Figure 3. Examples of Gene-Editing Strategies in Current Clinical Trials

(A) In cancer, gene-editing targets *PD-1* or *CD7* in T cells to enhance immune responses.

(B) In patients with HIV, *CCR5* is targeted in HSPCs to prevent HIV entry.

(C) In β-thalassemia and sickle cell disease, induction of fetal hemoglobin or correction of adult hemoglobin in HSPCs is used.

(D) AAV-mediated gene editing in the liver provides circulating enzymes in lysosomal storage diseases.

donor-derived T cells is being applied to generate universal CAR-T cells (Torikai and Cooper, 2016; Yang et al., 2015). This involves the disruption of the endogenous TCR to prevent graft-versus-host disease and of HLA components to prevent graft rejection by the host immune system.

HIV Infection/AIDS

In patients infected with HIV, T cells or HSPCs are harvested and the *CCR5* gene is knocked out *in vitro* by CRISPR/Cas9 or ZFNs before reinfusion into the patient (Holt et al., 2010; Perez et al., 2008; Tebas et al., 2014; Wang et al., 2014; Xu et al., 2017; Yu et al., 2018). As CCR5 is essential for HIV invasion of T cells, this intervention should prevent spreading of the virus into the engineered cells (Brelot and Chakrabarti, 2018).

Genetic Disorders

Sickle cell disease (SCD) and β-thalassemia are caused by disease-associated variants in the *HBB* gene, which encodes the beta subunit of hemoglobin. One strategy involves inducing the production of fetal

hemoglobin (HbF), which can compensate for malformed adult hemoglobin (in SCD) or reduced levels of adult hemoglobin (in β-thalassemia). This can be accomplished by *ex vivo* disruption of the intronic erythroid-specific enhancer of *BCL11A* in HSPCs by CRISPR/Cas9 or ZFN (Chang et al., 2017b; Psatha et al., 2018). After reinfusion, these HSPCs will produce erythrocytes with reduced BCL11A levels. As BCL11A represses transcription from *HBG* (Liu et al., 2018a), which encodes the gamma subunit of HbF, the level of the gamma subunit and thus HbF will increase. In another approach, one clinical trial is currently investigating direct gene restoration by CRISPR/Cas9 *ex vivo* in iPSC-derived HSPCs for the treatment of β-thalassemia (Cai et al., 2018; Martin et al., 2019; Park et al., 2019b; Wattanapanitch et al., 2018).

Clinical trials have started for the monogenic disorders hemophilia B and mucopolysaccharidosis (MPS) types I and II. Hemophilia B is caused by a deficiency of clotting factor IX, whereas MPS I and II are lysosomal storage disorders caused by deficiency of enzymes involved in the lysosomal degradation of glycosaminoglycans. These trials use systemic injection of AAV-expressing ZFN to achieve the targeted integration of a functional copy of the deficient gene into the albumin locus in liver cells, which secrete the enzyme into the circulation (Laoharawee et al., 2018; Sharma et al., 2015).

Finally, gene editing is being investigated in one of the subtypes of Leber's congenital amaurosis (LCA), a congenital retinal dystrophy. The subtype LCA10 is most commonly caused by an intronic variant in the *CEP290* gene that generates a cryptic splice site, resulting in defective protein production (Xu et al., 2018). A CRISPR/Cas9-based gene-editing strategy has been developed that removes the cryptic splice site; it is administered *in vivo* via subretinal injection using AAV as delivery vehicle (Maeder et al., 2019; Ruan et al., 2017).

FUTURE CHALLENGES

Among the most challenging aspects of gene editing to date are its delivery, specificity, and efficacy. The delivery strategy that is most appropriate for gene-editing intervention will depend on the cell-targeting approach that is most suitable for treating a specific disease. *Ex vivo* strategies can be conducted in a controlled environment, are amenable to efficient engineering using multiple methods, and may be a more straightforward way of bringing precision gene-editing medicine to the clinic. For example, when applied to stem cells (including adult stem cells or iPSCs) *ex vivo* gene editing can be subjected to quality control for genotoxicity before it is decided to engraft the cells in question. The use of *ex vivo* protein transduction ensures a transient exposure to nucleases, as opposed to the long-term exposure with *in vivo* approaches that use DNA constructs. However, *ex vivo* strategies face other issues associated with *ex vivo* cell manipulation. Engineered cells have to engraft efficiently into host individuals and should evade immune rejections if they are derived from a different donor. On the other hand, *in vivo* strategies will face the challenge of uncontrolled off-target events. In such cases, the selection of a suitable delivery vehicle, of a highly specific gene-targeting tool, and of possibly a strategy for spatiotemporal control, will all be especially relevant to preventing unintended variants and reducing potential immune reactions. *In vivo* gene-editing strategies that use persistent CAS9 expression—such as AAV—as a delivery vehicle may pose a risk of long-term exposure to gene-editing events, increasing the risk for off-target effects. Despite these challenges, the ongoing iteration of the CRISPR/Cas system into versions with improved specificity and efficacy holds great promise for a wide range of clinical applications. It will be important to remain cautious and, when the time comes for clinical testing, to evaluate the advantages against the possible risks. To fully understand the long-term effects of potential new treatments involving precise genomic engineering, thorough preclinical work is required.

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AUTHOR CONTRIBUTIONS

M.B., P.H.H., M.E., and W.W.M.P.P. wrote and revised the manuscript. M.B., P.H.H., and M.E. performed the graphical abstract and figures. A.T.v.d.P contributed to the review and editing of the manuscript. A.T.v.d.P and W.W.M.P.P. secured funding.

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DECLARATION OF INTERESTS

A.T.v.d.P. has provided consulting services for various industries in the field of Pompe disease under an agreement between these industries and Erasmus MC, Rotterdam, The Netherlands.

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Chapter 3

Ready for Repair? Gene Editing Enters the Clinic for the Treatment of Human Disease

Molecular Therapy
Methods & Clinical Development
Review

Ready for Repair? Gene Editing Enters the Clinic for the Treatment of Human Disease

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We present an overview of clinical trials involving gene editing using clustered interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9), transcription activator-like effector nucleases (TALENs), or zinc finger nucleases (ZFNs) and discuss the underlying mechanisms. In cancer immunotherapy, gene editing is applied *ex vivo* in T cells, transgenic T cell receptor (tTCR)-T cells, or chimeric antigen receptor (CAR)-T cells to improve adoptive cell therapy for multiple cancer types. This involves knockouts of immune checkpoint regulators such as PD-1, components of the endogenous TCR and histocompatibility leukocyte antigen (HLA) complex to generate universal allogeneic CAR-T cells, and CD7 to prevent self-destruction in adoptive cell therapy. In cervix carcinoma caused by human papillomavirus (HPV), E6 and E7 genes are disrupted using topically applied gene editing machinery. In HIV infection, the CCR5 co-receptor is disrupted *ex vivo* to generate HIV-resistant T cells, CAR-T cells, or hematopoietic stem cells. In β-thalassemia and sickle cell disease, hematopoietic stem cells are engineered *ex vivo* to induce the production of fetal hemoglobin. AAV-mediated *in vivo* gene editing is applied to exploit the liver for systemic production of therapeutic proteins in hemophilia and mucopolysaccharidoses, and in the eye to restore splicing of the CEP920 gene in Leber's congenital amaurosis. Close consideration of safety aspects and education of stakeholders will be essential for a successful implementation of gene editing technology in the clinic.

Conventional Gene Therapy

Traditionally, gene therapy relies on viral-based delivery of a protein-coding gene that either semi-randomly integrates into the genome (for retroviruses and lentiviruses) or remains as extrachromosomal DNA copy (for adeno-associated virus [AAV]).[1–3] These forms of gene therapy usually use overexpression of a protein that is missing or mutated in human disease. Lentiviral gene therapy has the advantage of being highly efficient and causing long-term efficacy. A drawback of lentiviral gene therapy is the lack of control of the location at which the virus integrates into the host genome, with the risk of insertional mutagenesis. By optimizing the lentiviral backbone and by controlling the number of viral copies, it has been demonstrated in multiple clinical trials that lentiviral gene therapy is safe provided that it is used with the proper precautions.[2,4] AAV-mediated gene therapy does not rely on integration into the host genome but instead involves delivery of a DNA episome to the nucleus. It is therefore considered to have a lower risk of genotoxicity compared to lentiviral gene therapy. However, episomal copies of AAV DNA are lost upon cell division, resulting in loss of efficacy. This restricts AAV gene therapy to nondividing cells. In addition, pre-existing immunity to AAV capsid proteins occurs in a significant percentage of the human population and precludes eligibility for the treatment.[5] Acquired immunity after a single AAV-mediated gene therapy treatment occurs invariably in patients and precludes eligibility for a second treatment. In both forms of gene therapy, cDNA overexpression can only be used when dosage effects of the transgene product do not apply. Although the desired average number of gene copies can be approached via the viral titer, it is not possible to precisely control this using viral-based overexpression.

Basics of Gene Editing

Developments in recent years have enabled the seamless engineering of the human genome using a variety of tools collectively termed gene editing. Precision gene editing strategies allow alteration of the genome of cells at specific loci to generate targeted genomic changes, which are being exploited for multiple applications in medicine. We first introduce the basics of gene editing and then summarize the major challenges for their clinical implementation. Gene editing tools that are currently under investigation in clinical trials include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered interspaced short palindromic repeats (CRISPR) in combination with CRISPR-associated protein (Cas). For a detailed comparison between these tools, we refer to previously published reviews.[6,7] In short, target site recognition occurs by sequence-specific DNA-binding proteins (in the case of ZFNs and TALENs) or by a short stretch of RNA termed single guide RNA (sgRNA; in the case of CRISPR-Cas). Current clinical applications of gene editing rely on the introduction of double-strand DNA breaks (DSBs), mediated by Fok-1 (in the case of ZFNs or TALENs) or by Cas nucleases (in the case of CRISPR-Cas) and the introduction of desired genomic alterations through the cell's endogenous DNA repair mechanisms.

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Two major DNA repair pathways are being exploited to conduct targeted genomic changes in clinical trials: (1) gene editing through homology-directed repair (HDR) used to replace a pathogenic variant or insert foreign DNA elements to restore the wild-type (WT) expression of a missing (or truncated) gene; and (2) non-homologous end joining (NHEJ) used to remove DNA elements leading to aberrant expression of genes or to gain a therapeutic function.

In contrast to traditional strategies for gene therapy, gene editing provides more versatile tools for gene therapy, for example to precisely correct point variants,[8,9] to place an extra, healthy gene copy at a safe genomic location of choice (a safe harbor: a location in the human genome at which integration of a gene is not harmful),[10,11] or to disrupt a gene. This would, for example, enable the restoration of endogenous expression levels following precise correction of the disease-associated variant within the natural locus, which would be especially important for gene products for which a correct dosage is required. It would also increase control of integration sites of a cDNA by choosing appropriate safe harbor locations. Such locations also should provide efficient transcription of the transgene by providing a favorable epigenetic environment consisting of euchromatin. Examples of safe harbor locations in the human genome are the albumin, *AAVS1*, and the *CCR5* loci.

On-Target or Off-Target?

Although the technology for gene editing is rapidly evolving, there are still important challenges for its clinical implementation. First, undesired editing of genomic regions can occur as a side effect of gene editing.[7] This can be off-target, i.e., the introduction of a DNA break outside the genomic region of choice due to the targeting of the gene editing machinery to a chromosomal location that carries sequence similarity to the region of interest. In this scenario, genes or regulatory regions other than the targeted gene can be modified, resulting in undesired downstream effects. Undesired events may include insertions, deletions, and chromosomal translocations.[12,13] Undesired variants can also be generated on-target, i.e., unintended modification of the genomic region of interest. In this scenario, regulatory elements within the gene of interest may be unintentionally changed. This may include elements involved in promoter activity, splicing, mRNA stability, protein translation, or microRNA (miRNA) genes (that are often present in introns or untranslated regions). The CRISPR-Cas9 system is inherently more prone to off-target effects compared to ZFNs or TALENs, because target site recognition in CRISPR-Cas9 relies on RNA-DNA interaction of only short stretches, and the RNA-DNA interaction allows some mismatches. In contrast, ZFNs and TALENs depend on highly specific protein-DNA interactions that allow fewer mismatches.[14] This has promoted much research directed toward enhancing the performance of CRISPR-Cas-based gene editing with respect to specificity and nuclease activity (see below). Methods to detect undesired events in gene editing often rely on *in silico* predictions, followed by analyses of predicted off-target events. This is not necessarily sufficient for clinical application, and unbiased analysis based on next-generation sequencing is expected to become an important tool in the future. For a more extensive discussion on off-target effects, see Broeders et al.,[7] Kim et al.,[15] Manghwar et al.,[16] and Pattanayak et al.[17]

Delivery of Gene Editing

The delivery of gene editing tools is a crucial aspect when it comes to clinical implementation. Two routes can be distinguished: *ex vivo* and *in vivo* delivery.[18,19] In *ex vivo* delivery, autologous or allogeneic cells are modified by gene editing outside the patient, and gene-modified cells are transplanted into the patient. Any route of administration of gene editing machinery can be applied *ex vivo*, such as transfection, nucleofection, or (viral) transduction. *Ex vivo* gene editing allows quality control prior to treatment. In particular, undesired off-target and on-target events can be monitored. Note that quality control can be performed on bulk generations of cells. Rare undesired events that occur in only a few cells and that might cause cellular transformation will be difficult to detect. Alternatively, this method involves an extra complication: the engraftment of (stem) cells. For example, maintaining engraftment potential and viability of the cell of interest can be challenging. Clinically, the most advanced forms of *ex vivo* gene editing involve T cells and hematopoietic stem cells (HSCs). In *in vivo* gene editing, gene editing tools are applied directly to the organism. Vehicles for delivery include AAV, lipid nanoparticles (LNPs), gold nanoparticles (GNPs), or cell-penetrating peptides (CPPs). The delivery method in *in vivo* gene editing is crucial for its safety.[20] When gene editing components are delivered *in vivo* via vehicles that remain present for an extended period, for example via AAV, there is a cumulative risk of undesired genotoxic events that can last for the time that the AAV remains present, which has been estimated to last for a period of 10 years or longer.[1] In contrast, when delivered as RNA or protein, there is only short-term exposure and a reduced risk of genotoxicity.

For *in vivo* gene editing, immunity against the delivery vehicle and the gene editing components are important considerations.[21] Both pre-existing and acquired immunity should be considered. The AAV delivery vehicle is subject to pre-existing immunity in a significant proportion of the population.[1] In addition, preexisting immunity to Cas9 protein from several species has been reported in several studies. This may neutralize the therapy or induce adverse events.[21–23]

In summary, the safety and efficacy of gene editing technology for the treatment of human disease depend on multiple factors, including the choice of the gene editing method, being either *ex vivo* or *in vivo*, the gene editing technique, target site selection, delivery method, and target tissue.

Gene Editing 2.0: Preclinical Developments

Technological developments are ongoing to improve gene editing tools with respect to specificity, efficiency, and versatility. These have been extensively described by us and others in recent reviews[7,24–26] and are only briefly mentioned here.

First, variations of the original CRISPR-Cas9 method have been designed. These include the following: homology-independent targeted integration (HITI) for generating a knockin via NHEJ without

involvement of HDR;[27] microhomology-mediated end joining (MMEJ)-dependent knockin, which is based on the presence of short stretches of homology that are utilized by the MMEJ DNA repair pathway;[28] base editing,[29] a mismatch repair- or base excision repair-dependent pathway in which a natural cytidine or adenosine deaminase (ADA) is coupled to a catalytically dead Cas9 (dCas9) to convert cytidine to uridine (which is replicated as thymidine), or to convert adenine to inosine, which is replicated as guanine; and prime editing,[30] in which a Cas9 nicking variant is used that introduces single stranded DNA breaks and that is coupled to reverse transcriptase to enable a wide variety of genomic changes. Second, other natural and engineered Cas9 variants have been identified and developed with distinct and/or enhanced targeting properties, including Cas12a (Cpf1), Cas12b (C2c1), FokI fused to dCas9,[31] Cas9-HF1,[32] eSpCs9,[33] evoCas9,[34] and HypaCas9.[35] Third, Cas9 variants with distinct protospacer-adjacent motif (PAM) recognition sites have been generated, including VQR and VRER variants, xCas9, and SpCas9-NG.[36] And fourth, sgRNAs have been modified with respect to their length, structure, and chemistry to reduce off-target properties.[37–39] These promising developments need future work to evaluate their suitability for clinical testing.

Scope of This Review

Whereas there have been numerous applications of gene editing in preclinical studies, information on clinical applications of gene editing is scattered in the literature. In this review, we present a comprehensive overview of current clinical trials using gene editing strategies for the treatment of human disease, and include selected preclinical examples. For more extensive overviews of preclinical studies, we refer to excellent reviews.[40,41] In addition, in this review, we focus on gene editing in somatic cells, and we refer to other recent reviews and opinion articles for editing the germline.[42–44] Thus far, precision gene editing has entered the clinic for the treatment of cancer immunotherapy, viral infections, and inherited hematologic, metabolic, and eye disorders (Table 1). These trials along with the underlying strategies are described in more detail below.

Gene Editing in Cancer Immunotherapy

Adoptive cell therapy (ACT) is a cellular form of cancer immunotherapy involving T cells with anti-tumor activity[45] that are expanded *ex vivo*, *ex vivo* genetically engineered or not, and applied to the patient via the circulation. Three major types of lymphocytes are used in ACT: (1) tumor-infiltrating lymphocytes (TILs), which are T cells that are isolated from tumors; and peripheral blood T lymphocytes that are (2) selected for tumor reactivity and expanded *ex vivo* before reinfusion or (3) genetically modified *ex vivo* with a transgenic T cell receptor (tTCR) or a chimeric antigen receptor (CAR) to target tumor cells.[46] ACT has been combined with *ex vivo* gene editing in a number of clinical trials, as discussed below.

Immune Checkpoint Knockout

Immune checkpoints are immune modulatory signals that can dampen the amplitude and quality of the immune response. Their physiological function is to prevent overstimulation of the immune system in order to maintain self-tolerance. A hallmark of cancer cells is their ability to exploit immune checkpoints to evade attack by the immune system. Cancer cells or their microenvironment can achieve this by activating immune checkpoints via overexpression of ligands or receptors that regulate the function of T cells.[47,48] In this way, cancer cells escape immune surveillance. To exploit this property of cancer cells for anti-cancer therapy, monoclonal antibodies have been developed that block natural immune checkpoints (present on T cells) or their ligands (present on cancer cells or in their micro-environment). This has revolutionized the field of anti-cancer therapy.[49] Examples include PD-1 and PD-L1 inhibitors, which have shown impressive results for treating different types of cancer at an advanced stage,[50,51] especially melanoma.[52] PD-1, encoded by the *PDCD1* gene, is a cell-surface receptor expressed on cytotoxic T cells that downregulates T cell activity upon interaction with its ligand PD-L1, which is overexpressed on malignant cells and cells in the tumor micro-environment.[48] In spite of general good tolerability, systemic administration of immune checkpoint inhibitors can result in autoimmune phenomena, referred to as immune-related adverse events (IRAEs).[53] IRAEs occur in up to 70% of patients receiving PD-1 and PD-L1 inhibitors[50,51] and have been described in multiple organ systems. Steroids might be used to manage IRAEs, but the extent of interference with immunotherapy is unknown.[53]

Knocking out immune checkpoint molecules in tumor-specific T cells is a promising strategy for ACT to circumvent systemic effects of checkpoint inhibition (Figure 1). When applied to total T cells harvested from patients, knocking out immune checkpoint molecules should render these less susceptible to immune inhibitory signals upon reinfusion. However, such an approach involves a heterogeneous T cell population rather than tumor-specific T cells. One solution to this problem would be to increase tumor specificity of circulating T cells *in vitro* by exposure to tumor-associated antigens.[54]

Due to the impressive clinical results from checkpoint inhibitors and TILs to treat melanoma, this type of cancer was chosen in the initial preclinical studies on applying immune checkpoint knockout (KO) in ACT using *ex vivo* gene editing. Promising *in vitro* results were reported from co-cultures of human tumor-specific T cells in which PD-1 was disrupted with melanoma cell lines,[55,56] and more recently by infusing PD-1 knockout T cells cells into mice that had been xenografted with human melanoma cells.[57] An improved cytotoxic effect of tumor-specific T cells following PD-1 knockout was also reported in preclinical studies of other cancer models, such as in a cultured gastric cancer cell line,[56] and in mice subcutaneously injected with either a fibrosarcoma cell line,[58] a multiple myeloma (MM) cell line,[59] or a liver cancer cell line.[60] Academic hospitals have been recruiting patients in clinical trials to investigate autologous, PD-1 knocked out T cells for the treatment of multiple types of cancer, including solid tumors arising from the esophagus,[61] lung,[62] prostate,[63] and Epstein-Barr-related neoplasms.[64] The publicly provided information is scarce. Presumably, as described for preclinical studies, these T cells have been manipulated *ex vivo* to enhance their tumor

Table 1. Current Clinical Trials Involving Gene Editing

Title	Tool	Status	Country	Delivery	ID	Ref.
Cancer Immunotherapy						
PD-1 knockout engineered T cells for advanced esophageal cancer	CRISPR-Cas9	completed	China	*ex vivo*	NCT03081715	61
PD-1 knockout engineered t cells for metastatic non-small cell lung cancer	CRISPR-Cas9	active, not recruiting	China	*ex vivo*	NCT02793856	62
Therapeutic vaccine plus PD-1 knockout in prostate cancer treatment	CRISPR-Cas9	recruiting	China	*ex vivo*	NCT03525652	63
PD-1 knockout EBV-CTLs for advanced stage Epstein-Barr virus (EBV) associated malignancies	CRISPR-Cas9	recruiting	China	*ex vivo*	NCT03044743	64
CD19 CAR and PD-1 knockout engineered T cells for CD19 positive malignant B cell derived leukemia and lymphoma	N.S.	not yet recruiting	China	*ex vivo*	NCT03298828	82
Study of PD-1 gene-knocked out mesothelin-directed CAR-T cells with the conditioning of PC in mesothelin positive multiple solid tumors	CRISPR-Cas9	recruiting	China	*ex vivo*	NCT03747965	83
CAR T and PD-1 knockout engineered T cells for esophageal cancer	N.S.	recruiting	China	*ex vivo*	NCT03706326	84
Anti-MUC1 CAR T cells and PD-1 knockout engineered T cells for NSCLC	N.S.	recruiting	China	*ex vivo*	NCT03525782	85
CRISPR (HPK1) edited CD19-specific CAR-T cells (XYF19 CAR-T Cells) for CD19$^+$ leukemia or lymphoma	CRISPR-Cas9	recruiting	China	*ex vivo*	NCT04037566	86
Study of UCART19 in pediatric patients with relapsed/refractory B acute lymphoblastic leukemia (PALL)	TALEN	active, not recruiting	US/EU/UK	*ex vivo*	NCT02808442	103
Dose escalation study of UCART19 in adult patients with relapsed/refractory B cell acute lymphoblastic leukaemia (CALM)	TALEN	active, not recruiting	US/EU/UK/Japan	*ex vivo*	NCT02746952	104
Safety and efficacy of ALLO-501 anti-CD19 allogeneic CAR T cells in adults with relapsed/refractory large B cell or follicular lymphoma (ALPHA)	TALEN	recruiting	US	*ex vivo*	NCT03939026	105
Safety and efficacy of ALLO-715 BCMA allogenic CAR T cells in in adults with relapsed or refractory multiple myeloma (UNIVERSAL)	TALEN	recruiting	US	*ex vivo*	NCT04093596	106
A study to evaluate the long-term safety of patients with advanced lymphoid malignancies who have been previously administered with UCART19/ALLO-501	TALEN	enrolling by invitation	US/EU/UK/Japan	*ex vivo*	NCT02735083	107
A study evaluating UCART019 in patients with relapsed or refractory CD19$^+$ leukemia and lymphoma	CRISPR-Cas9	recruiting	China	*ex vivo*	NCT03166878	112
A safety and efficacy study evaluating CTX110 in subjects with relapsed or refractory B cell malignancies	CRISPR-Cas9	recruiting	US/Australia/Germany	*ex vivo*	NCT04035434	115
A safety and efficacy study evaluating CTX120 in subjects with relapsed or refractory multiple myeloma	CRISPR-Cas9	recruiting	US/Australia	*ex vivo*	NCT04244656	116
CTA101 UCAR-T cell injection for treatment of relapsed or refractory CD19$^+$ B cell acute lymphoblastic leukemia	CRISPR-Cas9	recruiting	China	*ex vivo*	NCT04154709	117
Phase I study of UCART22 in patients with relapsed or refractory CD22$^+$ B cell acute lymphoblastic leukemia (BALLI-01)	TALEN	recruiting	US	*ex vivo*	NCT04150497	118

(*Continued on next page*)

Table 1. *Continued*

Title	Tool	Status	Country	Delivery	ID	Ref.
CTA101 in the treatment of relapsed or refractory diffuse large B cell lymphoma	CRISPR-Cas9	not yet recruiting	China	*ex vivo*	NCT04026100	119
A feasibility and safety study of universal dual specificity CD19 and CD20 or CD22 CAR-T cell immunotherapy for relapsed or refractory leukemia and lymphoma	CRISPR-Cas9	recruiting	China	*ex vivo*	NCT03398967	120
Study evaluating safety and efficacy of UCART123 in patients with acute myeloid leukemia (AMELI-01)	TALEN	recruiting	US	*ex vivo*	NCT03190278	121
Study evaluating safety and efficacy of UCART targeting CS1 in patients with relapsed/refractory multiple myeloma (MELANI-01)	TALEN	recruiting	US	*ex vivo*	NCT04142619	122
Anti-CD19 U-CAR-T cell therapy for B cell hematologic malignancies	N.S.	not yet recruiting	China	*ex vivo*	NCT04264039	123
Anti-CD7 U-CAR-T cell therapy for T/NK cell hematologic malignancies	N.S.	not yet recruiting	China	*ex vivo*	NCT04264078	124
Efficacy and safety evaluation of BCMA-UCART	N.S.	recruiting	China	*ex vivo*	NCT03752541	125
Safety and efficacy evaluation of CD19-UCART	N.S.	recruiting	China	*ex vivo*	NCT03229876	126
The clinical study of CD19 UCAR-T cells in patients with B cell acute lymphoblastic leukemia (B-ALL)	N.S.	recruiting	China	*ex vivo*	NCT04166838	127
NY-ESO-1-redirected CRISPR (TCRendo and PD1) edited t cells (NYCE T cells)	CRISPR-Cas9	terminated	US	*ex vivo*	NCT03399448	133
Study of CRISPR-Cas9 mediated PD-1 and TCR gene-knocked out mesothelin-directed CAR-T cells in patients with mesothelin positive multiple solid tumors	CRISPR-Cas9	recruiting	China	*ex vivo*	NCT03545815	134
Cell therapy for high risk T cell malignancies using CD7-specific CAR expressed on autologous T cells	CRISPR-Cas9	not yet recruiting	US	*ex vivo*	NCT03690011	144
Cervical Cancer						
Study of molecular-targeted therapy using zinc finger nuclease in cervical precancerous lesions	ZFN	N.S.	China	*in vivo*	NCT02800369	160
Study of targeted therapy using transcription activator-like effector nucleases in cervical precancerous lesions	TALEN	N.S.	China	*in vivo*	NCT03226470	161
A safety and efficacy study of TALEN and CRISPR/Cas9 in the treatment of HPV-related cervical intraepithelial neoplasia	CRISPR-Cas9 TALEN	N.S.	China	*in vivo*	NCT03057912	162
HIV Infection and AIDS						
Autologous T cells genetically modified at the CCR5 gene by zinc finger nucleases SB-728 for HIV	ZFN	completed	US	*ex vivo*	NCT00842634	189
Phase 1 dose escalation study of autologous t cells genetically modified at the CCR5 gene by zinc finger nucleases in HIV-infected patients	ZFN	completed	US	*ex vivo*	NCT01044654	190
Repeat doses of SB-728mR-T after cyclophosphamide conditioning in HIV-infected subjects on HAART	ZFN	completed	US	*ex vivo*	NCT02225665	191
A phase I study of T cells genetically modified at the CCR5 gene by zinc finger nucleases SB-728mR in HIV-infected patients	ZFN	completed	US	*ex vivo*	NCT02388594	192
Dose escalation study of cyclophosphamide in HIV-infected subjects on HAART receiving SB-728-T	ZFN	completed	US	*ex vivo*	NCT01543152	193

(*Continued on next page*)

Table 1. *Continued*

Title	Tool	Status	Country	Delivery	ID	Ref.
CCR5-modified CD4$^+$ T cells for HIV infection (TRAILBLAZER)	ZFN	recruiting	US	*ex vivo*	NCT03666871	194
Study of autologous T cells genetically modified at the CCR5 gene by zinc finger nucleases in HIV-infected subjects	ZFN	completed	US	*ex vivo*	NCT01252641	195
Long-term follow-up of HIV subjects exposed to SB-728-T or SB-728mR-T	ZFN	enrolling by invitation	US	*ex vivo*	NCT04201782	197
Safety study of zinc finger nuclease CCR5-modified hematopoietic stem/progenitor cells in HIV-1 infected patients	ZFN	active, not recruiting	US	*ex vivo*	NCT02500849	203
Safety of transplantation of CRISPR CCR5 modified CD34$^+$ cells in HIV-infected subjects with hematological malignances	CRISPR-Cas9	recruiting	China	*ex vivo*	NCT03164135	204
CD4 CAR+ ZFN-modified T cells in HIV therapy	ZFN	active, not recruiting	US	*ex vivo*	NCT03617198	206
β-thalassemia and Sickle Cell Disease						
A safety and efficacy study evaluating CTX001 in subjects with transfusion-dependent β-thalassemia	CRISPR-Cas9	recruiting	US/Canada/EU/UK	*ex vivo*	NCT03655678	263
A study to assess the safety, tolerability, and efficacy of ST-400 for treatment of transfusion-dependent beta-thalassemia (TDT)	ZFN	active, not recruiting	US	*ex vivo*	NCT03432364	264
A safety and efficacy study evaluating CTX001 in subjects with severe sickle cell disease	CRISPR-Cas9	recruiting	US/Canada/EU	*ex vivo*	NCT03745287	265
A study to assess the safety, tolerability, and efficacy of BIVV003 for autologous hematopoietic stem cell transplantation in patients with severe sickle cell disease (BIVV003)	ZFN	recruiting	US	*ex vivo*	NCT03653247	266
A long-term follow-up study in subjects who received CTX001	CRISPR-Cas9	enrolling by invitation	US/EU	*ex vivo*	NCT04208529	267
iHSCs with the gene correction of HBB intervent subjests with β-thalassemia mutations	CRISPR-Cas9	not yet recruiting	N.S.	*ex vivo*	NCT03728322	280
Hemophilia						
Ascending dose study of genome editing by zinc finger nuclease therapeutic SB-FIX in subjects with severe hemophilia B	ZFN	active, not recruiting	US	*in vivo*	NCT02695160	289
Mucopolysaccharidoses						
Ascending dose study of genome editing by the zinc finger nuclease (ZFN) therapeutic SB-318 in subjects with MPS I	ZFN	active, not recruiting	US	*in vivo*	NCT02702115	319
Ascending dose study of genome editing by the zinc finger nuclease (ZFN) therapeutic SB-913 in subjects with MPS II	ZFN	active, not recruiting	US	*in vivo*	NCT03041324	320
Leber's Congenital Amaurosis						
Single ascending dose study in participants with LCA10	CRISPR-Cas9	recruiting	US	*in vivo*	NCT03872479	329

N.S., not specified.

specificity, but this has not been specified. Recently, the results for PD-1-edited T cells in metastatic lung carcinoma patients were published.[65] Although no methods for increasing the tumor specificity of T cells was described, no severe adverse events were reported in 12 patients after a median follow-up time of 47.1 weeks. Despite the treatment, 10 patients progressed, and only 2 responded transiently. Although not designed to investigate the therapeutic effect, these results were somewhat disappointing and are possibly caused by inadequate levels of tumor-specific T cells.

Another method of generating tumor-specific T cell clones is the *ex vivo* expansion of T cells that are isolated from tumor tissue,

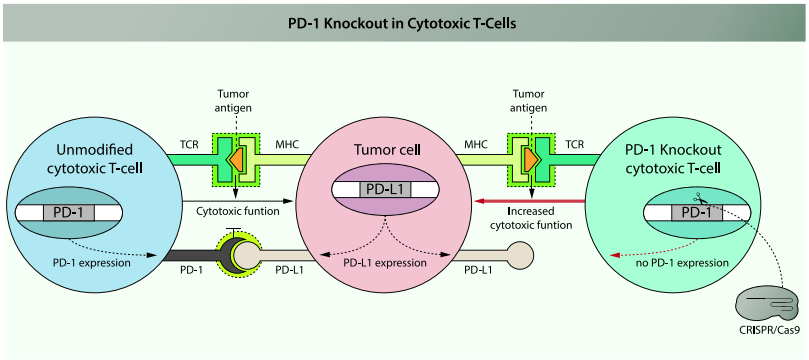


Figure 1. Effect of PD-1 Knockout in Cytotoxic T Cells

Cytotoxic T cells are able to recognize tumor cells via the T cell receptor (TCR). This receptor recognizes an antigen that is presented on potential target cells by the MHC. Binding results in T cell activation through signal transduction. The activated T cell will expand and exert its cytotoxic effector function on target cells, thus inducing apoptosis. If the target cell expresses PD-L1, it can interact with PD-1 that is expressed on the surface of the T cell. This will lead to activation of PD-1, one of the immune checkpoint molecules, resulting in inhibition of the T cell's cytotoxic activity. If *PD-1* is disrupted in the cytotoxic T cell, PD-L1 expressed from the tumor cell can no longer interact with the T cell and inhibition of T cell cytotoxicity is prevented. In this scenario, *PD-1* disruption prevents escape of tumor cells from attack by cytotoxic T cells. Red indicates the result of intervention.

so-called TILs. Although not yet clinically applied, PD-1 knockout in TILs has resulted preclinically in an improved anti-tumor effect *in vitro*[55] and *in vivo*.[58]

Innate immune cells such as dendritic cells (DCs) and natural killer (NK) cells are also target cells for the development of immunotherapy against cancer.[66] Importantly, NK cells have also been shown to express several immune checkpoint inhibitors.[67] An example of recent preclinical developments is the knockout of the *NKp46* and *CIS* checkpoint genes in primary human NK cells.[68,69] Although gene-edited innate immune cells have not yet reached clinical trials, these efforts illustrate the ongoing work that might promote their clinical development.

Immune Checkpoint Knockout in Genetically Engineered T Cells: tTCR-T and CAR-T cells

Besides the isolation of T cells with enhanced anti-tumor activity from patients, it is also possible to induce tumor specificity in T cells using genetic engineering (using viral transduction or gene editing). Such redirected T cells can be generated by forced expression of receptors with enhanced specificity for a tumor-associated antigen, such as tTCRs or CARs.[70,71] tTCRs are transgenic forms of naturally occurring receptors isolated from tumor-specific T cells and depend on the major histocompatibility complex (MHC) for efficient antigen recognition.[72] CARs are synthetic receptors that do not depend on MHC for efficient antigen binding.[73] To avoid negative regulation by tumors, immune checkpoint inhibition (using antibodies) or knock out (using gene editing) are also worthwhile strategies in tTCR-T cells and CAR-T cells.

The concept of immune checkpoint knockout in redirected T cells has been demonstrated *in vitro* and *in vivo*, both for tTCR-T cells[74] and CAR-T cells.[75–79] Improved antitumor reactivity of redirected T cells after PD-1 disruption was observed in a range of preclinical cancer models, for example, models of melanoma,[74] hepatocellular carcinoma,[75] glioma,[76,79] breast cancer,[77] and erytroleukemia.[78] In addition, encouraging clinical results have already been obtained by combining CAR-T cells with immune checkpoint inhibitors.[80,81] Using gene editing, PD-1 knockout in CAR-T cells that were redirected against the B cell marker cluster of differentiation 19 (CD19)[82] and membrane proteins mesothelin[83] and MUC1,[84,85] which are upregulated in a range of malignancies, are investigated in clinical trials for the treatment of B cell leukemia/lymphoma,[82] multiple mesothelin-positive solid tumors (such as pancreatic cancer, cholangiocarcinoma cancer, and ovarian cancer),[83] esophageal cancer,[84] and lung cancer.[85] One trial investigates the infusion of CAR-T cells carrying an HPK1 knockout in patients with relapsed or refractory $CD19^+$ leukemia or lymphoma.[86] HPK1 is a protein kinase that was found to suppress the anti-tumor response of T cells by attenuating TCR signaling.[87] In addition, HPK1 exerts T cell inhibitory effects downstream of E prostanoid receptor activation by prostaglandin E2, a metabolic byproduct that is overproduced by cancers such as non-small-cell lung carcinomas.[88,89] Mice with a kinase-dead HPK1 showed improved anti-tumor[89,90] and antiviral responses.[90]

Disruption of other molecules with immunomodulatory effects in ACT has been performed in preclinical studies, but no clinical trials are currently open. For example, infusion of cytotoxic T cells in which the immune checkpoint gene *CTLA-4* was disrupted resulted in decreased tumor growth compared to infusion of non-edited

counterparts in mice that were xenografted subcutaneously with bladder cancer cell lines[91] or colon cancer cell lines.[92] In addition, the anti-tumor effect of CAR-T cells against a human glioma cell line that was subcutaneously engrafted in mice was enhanced upon knockout of *DGK*,[93] which encodes an intracellular enzyme that negatively regulates TCR signaling.[94] In contrast, disruption of the immune checkpoint gene *LAG-3* in CAR-T cells did not result in an enhanced anti-tumor effect in mice subcutaneously engrafted with a human lymphoma cell line,[95] suggesting that the choice of immune checkpoint gene is important to design an efficient treatment.

Universal ACT

So far, we discussed autologous T cell therapies. However, this is not always feasible for every patient.[96] The establishment of universal, allogeneic ACT might be an attractive alternative, because such "off-the-shelf" therapy would overcome the high costs and experimental burden of manufacturing a custom-made autologous or histocompatibility leukocyte antigen (HLA)-matched allogeneic therapy for every patient. For such therapy, the risks of graft-versus-host disease (GvHD) and graft rejection by the patients' immune system for universal ACT must be addressed. The strategies used involve knockout of the TCR to prevent GvHD, and knockout of human leukocyte antigen (HLA) genes to prevent graft rejection by the host immune system.[97,98] Clinical studies and preclinical examples are discussed below.

In vitro studies showed that anti-CD19 CAR-T cells, which target B cells, tolerated ZFN-mediated knockout of the TCR, as assessed by cell proliferation and their ability to lyse target cells.[99] In addition, *in vivo* application of such cells demonstrated an anti-leukemic response in mice that were intravenously injected with a lymphoma cell line that was similar or better compared to non-edited cells.[100,101] The feasibility of clinical implementation of such a strategy was illustrated by a study in which two therapy-refractory pediatric patients with acute lymphoblastic leukemia (ALL) were treated with allogeneic anti-CD19 CAR-T cells from unselected donors[102] that had been engineered *in vitro* using TALENs in two ways. First, expression of the endogenous αβ TCR was disrupted by targeting the constant region of the TCR α chain. Second, *CD52* was knocked out with the following rationale. CD52 is expressed on T cells, and anti-CD52 antibodies (alemtuzumab) are part of the conditioning regimen prior to allogeneic HSC transplantation to reduce the risk of graft rejection by the host's lymphocytes. To prevent alemtuzumab from attacking anti-CD19 CAR-T cells, these cells were made resistant by knockout of *CD52*. Despite development of grade 2 GvHD in one of the patients, the results of this trial indicated an ongoing disease-free survival of the two patients of 12 and 18 months after the start of therapy.[102] These results suggest that off-the-shelf allogeneic CAR-T cells therapy is feasible, and that adverse events such as GvHD are manageable. This exact strategy is adopted in clinical trials investigating universal CAR-T cells in pediatric or adult B cell ALL,[103,104] B cell lymphoma,[105] and MM patients.[106] The long-term effects of two of these products are investigated in a separate trial.[107]

To reduce the risk of graft rejection by the host immune system, *HLA* genes have been disrupted in donor T cells.[108–111] Notably, CRISPR-Cas9-mediated triple KO of the T cell receptor α constant (*TRAC*) *locus*, an HLA complex gene (*B2M*), and an immune checkpoint gene (*PDCD1*) was used to potentiate the anti-tumor effect of CAR-T cells against multiple targets in mouse models, for example in mice intravenously injected with a B cell ALL cell line,[109] in mice intraperitoneally injected with a lymphoma cell line,[110] and in mice intracerebrally injected with a glioma cell line.[111] In one active clinical trial both the endogenous TCR and HLA complex are knocked out in anti-CD19 CAR-T cells for treating of B cell leukemia and lymphoma.[112]

In another concept, a tumor-targeting CAR or tTCR is inserted into the *TRAC* locus using CRISPR-Cas9-mediated HDR. This yields two effects: knockout of the endogenous TCR, and knockin of the CAR/tTCR. In a preclinical study, a CD19-directed CAR was inserted into the *TRAC* locus in human T cells by HDR using CRISPR-Cas9.[113] When these CAR-T cells were administered to a mouse model of ALL, an improved anti-leukemic response was observed that resulted in prolonged survival compared to conventionally generated CAR-T cells.[113] A similar strategy proved feasible for inserting a tTCR directed against the immunogenic cancer antigen NY-ESO-1 in the *TRAC* locus.[114] This strategy is adopted in two clinical trials for patients with B cell malignancies[115] or MM,[116] in which the endogenous TCR is disrupted by knockin of an anti-CD19 or anti-BCMA CAR in the TCR locus of allogeneic T cells, respectively. In addition, the HLA complex is disrupted by knockout of the B2*M* gene.

Additional clinical studies are planned, in which infusion of universal CAR-T cells (engineered using TALENs or CRISPR-Cas9) will be investigated for the treatment of B cell ALL or lymphoma,[117–120] acute myeloid leukemia (AML),[121] and multiple myeloma.[122] No molecular details are provided for these trials. Five more clinical trials are active or planned that will investigate universal CAR-T cells in hematological malignancies, but no information on the applied gene editing platform has been provided.[123–127]

A challenging application in one of the aforementioned trials is the treatment of AML with ACT, because molecular targets of leukemic cells in AML are also expressed in HSCs. As a result, ACT will attack the host's HSCs and impair hematopoiesis.[128] Indeed, severe myelotoxicity, leading to prolonged pancytopenia, was seen in preclinical studies using CAR-T cells directed at CD33[129] and CD123.[130] One possible strategy to circumvent this problem would be to co-transplant HSCs in which the target molecule is knocked out together with the CAR-T cells. As the CAR-T cells will attack the leukemic cells and unmodified recipient HSCs, the gene-edited donor HSCs will not be targeted anymore and will repopulate the bone marrow. This strategy has been proven feasible in a mouse model for AML, in which anti-CD33 CAR-T cells along with CD33-edited HSCs were used.[131] The leukemic cells responded to anti-CD33 CAR-T cell treatment, while myelotoxicity was selectively mitigated in mice transplanted with CD33-edited HSCs. An ongoing clinical trial investigates universal CAR-T cells in refractory or relapsed AML, but it does not include a method to mitigate the possible myelotoxic effect of CAR-T cells.[121]

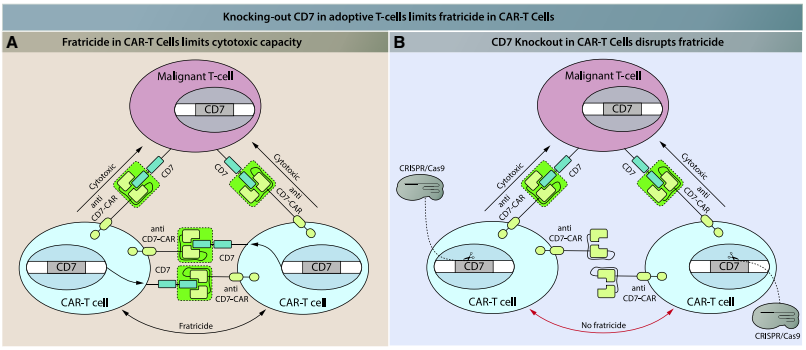


Figure 2. CD7 Knockout in Anti-CD7 CAR-T Cells Prevents Fratricide

(A) Anti-CD7 CAR-T cells recognize the CD7 antigen on (malignant) T cells via their chimeric antigen receptor, which triggers the CAR-T cell cytotoxic function and thus results in lysis of the target cell. CD7 is expressed on the surface of all T cells. As CAR-T cells also express CD7, CAR-T cells will recognize other CAR-T cells and lyse these, which is termed fratricide. (B) The gene encoding CD7 can be knocked out in anti-CD7 CAR-T cells, for example by CRISPR-Cas9. Without CD7, these CAR-T cells will not be recognized and lysed by other anti-CD7 CAR-T cells, thus preventing fratricide. Red indicates the result of intervention.

Endogenous TCR Knockout in Autologous ACT

Above we described the knockout of the endogenous TCR in allogeneic ACT products to prevent GvHD. However, there is also a rationale for knocking out endogenous TCR components in autologous ACT. The reason for this is that the endogenous TCR can interfere with the function of the tTCR/CAR, either by competing for cell surface expression, or by dimerization to form a novel hybrid compound TCR that might cause autoimmune reactions.[132] Knockout of endogenous TCR components in autologous ACT cells is therefore adopted in two clinical trials with either tTCR-T cells redirected against NY-ESO-1 (in MM, melanoma, or subtypes of sarcoma)[133] or CAR-T cells redirected against mesothelin (in any mesothelin-positive solid tumor).[134] PD-1 is also knocked out in the tTCR-T cells and CAR-T cells in these trials. Initial results of the first trial have been published, and they indicated no major adverse events in the three patients that were included.[135] The patients suffered from advanced refractory malignancies, and the response to therapy was variable: one patient did not respond and died, while two patients showed initial disease stabilization, followed by disease progression after 30 or 100 days. Responses to follow-up treatment in these two patients were variable. Interestingly, the authors reported a relatively long half-life of tTCR-T cells at an average of 83.9 days. As other studies reported a half-life of roughly 1 week of non-edited NY-ESO-1 tTCR-T cells,[136–138] the knockout of PD-1 and/or endogenous TCR components might have contributed to a slower decay of the tTCR-T cells.

A Special Case: ACT for T Cell Malignancies

It is particularly challenging to design an effective ACT using T cells for T cell malignancies. T cells should target molecules that are preferably expressed by malignant T cells but not by normal T cells. The difficulty in finding specific targets in malignant T cells results in self-destruction of tTCR-T cells or CAR-T cells cells used in ACT.[139] This process, called fratricide, can interfere with ACT efficacy and has been observed in both CAR-T cells[140] and transgenic TCR-T cells.[141] One possible solution to this problem is to knockout the target molecule in the adoptive T cells by gene editing. In this way, transgenic TCR-T or CAR-T cells will recognize and attack malignant T cells, but not each other. This strategy has been proven effective in circumventing fratricide in a preclinical setting,[142,143] and it is currently applied in a clinical trial applied to CD7. CD7 is expressed on the cell surface of T cells, and in this trial anti-CD7 CAR-T cells are tested for the treatment of T cell leukemia/lymphoma. To prevent fratricide, CD7 was knocked out in CAR-T cells using CRISPR-Cas9 (Figure 2).[144] In addition, one previously mentioned clinical trial investigates universal anti-CD7 CAR-T cells in T cell malignancies, but knockout of CD7 in the CAR-T cells has not been mentioned.[124]

Gene Editing in Viral Infection

Cervical Cancer

Cervical cancer is the third most prevalent type of cancer in women worldwide.[145] The most contributing etiological factor is human papillomavirus (HPV) infection via sexual intercourse, especially serotypes HPV-16 and HPV-18. Most HPV infections are cleared by the host immune system, but persistent infections can give rise to malignant transformation. Several vaccines have been developed for primary prevention of cervix carcinoma, with varying levels of population coverage worldwide.[146] Premalignant lesions are treated by local excision, while therapeutic modalities for invasive cervix carcinoma are dependent on the cancer stage and include surgery, radiotherapy, and chemotherapy.[147] In spite of these preventive and curative modalities, survival rates of cervical cancer range from 93% at early disease stage to 15% at disseminated disease stage.[148] New

treatment modalities are crucial to increase survival rates of cervix carcinoma.

One such recent advance is RNA interference (RNAi)-mediated knockdown of the viral oncogenes E6 and E7, as these have been identified to drive and sustain HPV-related carcinogenesis.[149] In multiple studies, knockdown of E6 and E7 resulted in increased cell death in HPV-positive cell lines.[150,151] However, multiple obstacles, such as the occurrence of escape mechanisms and insufficient efficiency, have prohibited RNA-based strategies from entering clinical trials so far.[152] Guided gene knockout might partially overcome these limitations. First of all, RNAi only lowers target gene expression, whereas gene editing can completely disrupt or delete a gene, leaving no room for residual gene expression. Mutation of the target region, a known escape mechanism of RNA viruses, as observed in studies using RNAi-mediated knockdown, likely still applies to knockout strategies using gene editing. Another escape mechanism, which is expression of viral suppressors of RNAi, is expected not to apply to gene editing.[153] Investigating viral escape from strategies involving gene editing in cervical cancer caused by HPV will be an important aspect in future research. As is true for any cancer, it will be important to start treatment at the earliest stage possible and to use treatments that are highly efficient.

Gene editing for treating HPV infection has focused on *E6* and *E7*. It is generally appealing to target viral genes, because these are exogenous sequences, reducing the chances of unintended off-target events in endogenous genes. Successful knockout of *E6* and *E7* genes has been achieved via ZFNs,[154] TALENs,[155,156] and CRISPR-Cas9.[157–159] The *in vitro* knockout of viral *E6* or *E7* sequences in HPV-infected cell line models caused inhibition of cell growth and cell viability, which is in line with results obtained from RNAi. In addition, gene-edited cells showed reduced capability to engraft in mice compared to unedited cells when transplanted subcutaneously.[154,155,157] Results were consistent for targeting HPV-16 and HPV-18.[155] Furthermore, *in vivo* gene editing with topically applied TALEN components using polymer-complexed T512 plasmids in K14-HPV16 transgenic mice, a model system for cervical HPV-16 infection, resulted in reduced viral DNA loads and a reversal of histological malignant abnormalities.[155] As only the TALEN platform was topically applied *in vivo* in a cervical cancer mouse model,[155] the effects of topically applied gene editing tools on cervical cancer could not be compared. Based on these results, multiple clinical trials have been designed to investigate gene editing of precancerous cervical lesions, directed at the HPV genome. These clinical trials apply either ZFN,[160] TALEN,[161,162] or CRISPR-Cas9[162] gene editing platforms, which are administered either by topical gel or vaginal suppository.

In the future, topically applied gene editing tools might be investigated in combination with chemotherapy in metastasized cervix carcinoma. Preclinically, an additive anti-cancer effect of gene editing was already shown *in vitro* and *in vivo* in combination with cisplatin.[163] In addition, the potential of HPV targeting extends beyond the treatment of cervix carcinoma, as HPV-related cancers include other anogenital cancers such as vulvar, vaginal, anal, and penile cancer, but also cancers in the head and neck region.[164] In preclinical studies, CRISPR-Cas9-based strategies have been tested for treating other chronic viral infections, such as hepatitis B virus,[165–172] Epstein-Barr virus,[173–176] and human immunodeficiency virus (HIV) (see section below). As these viral infections affect distinct tissues and/or have distinct modes of action, these might need tailored strategies for delivery to the required target. An overview of these gene editing strategies is provided in a review by de Buhr and Lebbink.[177]

Gene Editing in HIV Infection and AIDS

HIV is a lentivirus that integrates its genome (after reverse transcription of its RNA into DNA) into the genome of host $CD4^+$ T helper cells, forming a provirus. After the initial acute phase of infection, a pool of T cells remains latently infected. When the provirus becomes activated, host cells produce new viral particles and undergo cell death. This causes acquired immunodeficiency syndrome (AIDS) if the numbers of T helper cells drop to levels that are insufficient to effectively protect the host from infections or malignant transformations.[178] Currently, HIV infections are treated by antiretroviral therapy (ART) to reduce the risk of progression to AIDS. However, ART needs to be taken life-long, requires adherence to the treatment regimen, and can have side effects and incomplete efficacy.[179,180] Although no curative treatment has been found to date, there are two documented cases of HIV patients who have been cured from HIV infection. The first patient, known as the Berlin patient, received two HSC transplantations for AML, and has remained HIV-negative since.[181,182] His donor harbored a homozygous *CCR5* Δ32/Δ32 loss-of-function allele, which had previously been known to impair infection of T cells by HIV-1.[183] A similar second patient was identified recently.[184] In addition, genetic association studies have shown that *CCR5Δ32* homozygotes are resistant to HIV infection, whereas heterozygotes display delayed progression of disease.[185–187] It was therefore hypothesized that *ex vivo* disruption of *CCR5* in patient-derived T cells, followed by reinfusion, could mimic the curative outcome of the Berlin patient. *CCR5* was targeted by ZFNs in human primary $CD4^+$ T cells, and biallelic gene disruption was achieved in 33% of modified cells *in vitro*.[188] In an HIV infection mouse model, injection of *CCR5* KO T cells resulted in decreased viral load and an increased T cell population compared to wild-type T cells.[188] Six out of a total of seven clinical trials assessing the infusion of autologous $CD4^+$ *CCR5* knockout T cells using ZFNs have been completed,[189–195] and results of one have been published.[196] In the study of Tebas et al.,[196] $CD4^+$ CCR5 KO T cell infusion proved to be safe in HIV patients. In addition, levels of blood HIV DNA decreased in most patients, although the trial was not designed to measure efficacy. One clinical trial is currently investigating the long-term effects of *CCR5*-edited T cells.[197]

It is unclear how long engineered T cells can in principle protect against AIDS given their limited lifespan. Therefore, several groups are focusing on deleting *CCR5* in HSCs, as these have self-renewal capacity to remain present as stem cells and can give rise to all cells of

the hematopoietic lineage.[198] HSCs would for example also give rise to $CD4^+$ myeloid cells, which are also susceptible to HIV infection.[199] *CCR5* disruption by ZFNs was achieved in human $CD34^+$ HSCs, and these cells were able to engraft in immunosuppressed or immunodeficient mice.[200–202] In addition, infusion of *CCR5*-modified HSCs resulted in reduced plasma HIV levels in mouse models when compared to unmodified HSC infusions.[202] Currently, two clinical trials are recruiting patients to test this strategy using either ZFN[203] or CRISPR-Cas9.[204]

The previous strategies involve supplying patients with HIV-resistant cells to diminish the effect of HIV on the immune system. Alternatively, CAR-T cells that are redirected toward HIV-related proteins can be applied to actively attack T cells that are infected by the virus.[205] Via gene editing, *CCR5* might be disrupted in the CAR-T cells to prevent HIV from infecting these cells. Multiple clinical trials are planned or ongoing for CAR-T cells as a treatment option for HIV. In one of those, ZFNs are applied to disrupt *CCR5* in CAR-T cells.[206]

CCR5 disruption will not be efficacious in all patients, since CCR5 might be redundant for cell entry by certain HIV strains.[207,208] Another disadvantage is the necessity of biallelic knockout of *CCR5* to efficiently impair viral reproduction.[198,209] An alternative is disruption of the HIV genome itself, which may be especially attractive since this is not an endogenous sequence and may therefore be less susceptible to off-target effects. Targeted disruption of the HIV genome, however, faces the challenge of mutational escape. Another challenge is that HIV-1 forms a stable reservoir in resting $CD4^+$ T cells, which sustains the disease and causes the residual viremia in patients undergoing ART.[210] If the latent reservoir could be directly targeted or activated, HIV infection could possibly be cured without the requirement of myeloablative therapy and subsequent HSC transplantation. Multiple proof-of-principle studies have shown the feasibility of targeting HIV genomic sequences in infected cells *in vitro*,[211–219] but the problems of mutational escape and targeting the HIV latent reservoir have not been solved to date.[220]

Alternatively, the strategies mentioned above could be realized via RNAi. *CCR5* knockdown by short hairpin RNA (shRNA) in HSCs or T cells has been readily tested in preclinical studies and is the subject of a phase I/II clinical trial.[221] Targeting of HIV transcripts by RNAi has also been tested preclinically.[221] Besides mutational esacape mentioned above, RNAi faces the additional challenge of transcriptional upregulation of the target in response to knockdown.[222]

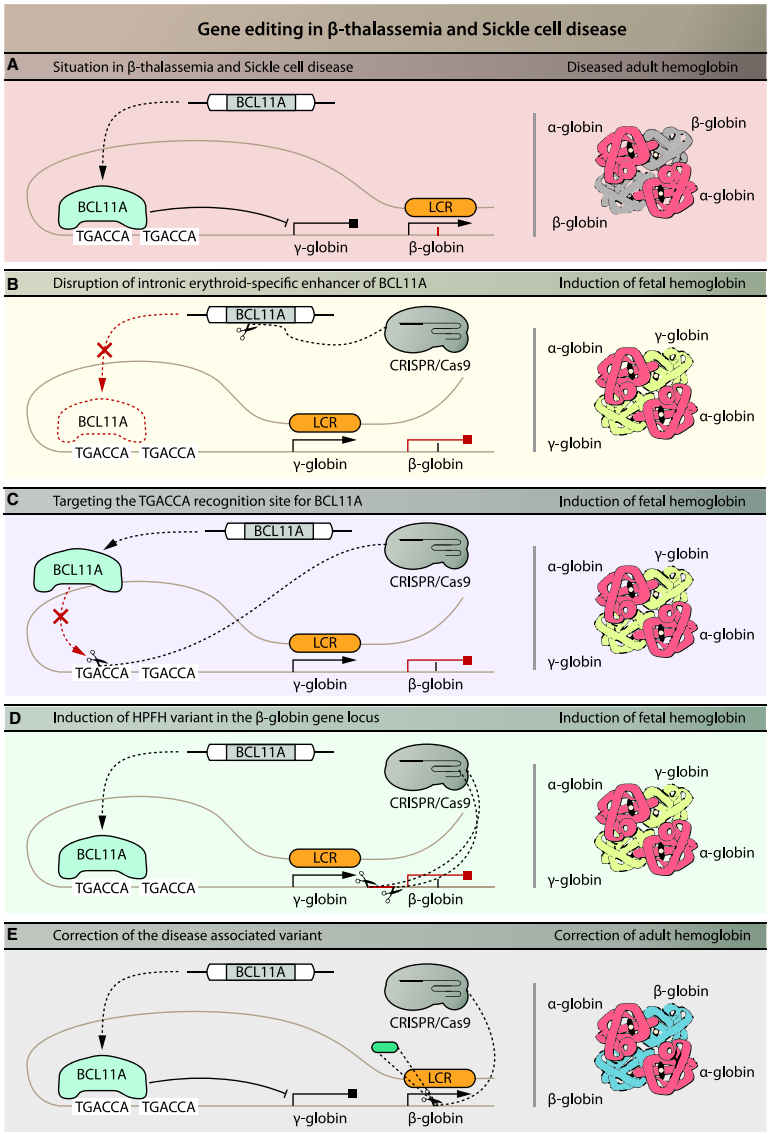
Exciting preclinical studies have shown the feasibility for applying gene editing to the engineering of B cells that produce antibodies specific to a number of viruses, including Rous sarcoma virus (RSV), influenza virus, Epstein-Barr virus (EBV), or HIV, all of which are viruses for which there is to date no vaccine available. In the example of HIV, broad neutralizing antibodies (bNAbs) have been detected in a small number of infected individuals at ~1–3 years after infection.[223] These NAbs protect against HIV infection. Primary human B cells have been successfully engineered using CRISPR-Cas9 to produce NAbs against HIV,[224] and a proof of principle using engineered mouse B cells provided protection against infection with RSV.[225]

Gene Editing in Hematological Disorders

β-thalassemia and Sickle Cell Disease

β-thalassemia is an autosomal recessive disease with more than 200 known disease-associated variants in the gene coding for the hemoglobin β chain (*HBB*), resulting in a clinically variable phenotype. All of these variants cause reduced or abolished translation of the HBB protein.[226] Approximately 98% of total adult hemoglobin is composed of hemoglobin A (HbA), which is formed by two β-globin subunits bound to two α-globin subunits.[227] Reduced expression of the β-globin subunit results in a relative excess of the α-globin subunit, resulting in precipitation of the α-globin subunit in erythroblasts and erythrocytes. This ultimately leads to impaired erythropoiesis and hemolysis, and thus anemia.

Treatment of β-thalassemia depends on life-long supportive measures, of which blood transfusion is the main component. β-Thalassemia patients either have transfusion-dependent thalassemia (TDT) or non-TDT (NTDT).[228] TDT patients require life-long blood transfusions for survival, starting at an average age of 2 years for every 2–5 weeks, while NTDT patients need blood transfusions only occasionally or for limited periods of time.[228] Regular transfusions place patients at risk of blood-borne infections, iron overload, and transfusion reactions.[229] In addition, 80% of TDT patients develop long-term complications.[230] Although long-term complications due to iron overload result in decreased longevity, a life expectancy of over 50 years of age has been estimated.[231] Recurrent therapy, adverse events, and complications also negatively impact patients quality of life. Furthermore, treatment of β-thalassemia patients with iron chelation therapy is essential to reduce iron overload, but it results in considerable additional costs. In addition, through alloimmunization, it becomes increasingly challenging to find eligible blood products.[229] The only curative therapy to date is allogeneic HSC transplantation, provided that a suitable donor is available. An HLA-matched sibling donor is available in about 30% of cases.[232] For the remaining patients an unrelated HLA-matched donor should be considered, which approaches success rates of sibling donors. However, for 20%–30% of patients needing an HSC transplantation (without considering the underlying disease), no optimal unrelated HLA-matched donor can be found even with the extensive donor registries that have been established in Europe.[233] For 5% of patients, no donor could be identified at all. The alternative of cord blood transplantation from unrelated donors, for which HLA matching is less stringent, is less favorable due to higher rates of graft failure.[234] Haploidentical, or half-matched (e.g., parents or children), stem cell transplantation seems inferior to HLA-matched unrelated transplantation due to delayed restoration of the immune system, although experience is limited.[232] Between 2000 and 2010, the European Society for Blood and Bone Marrow Transplantation Hemoglobinopathy Registry reported treatment outcomes for all HSC transplantations, showing a 2-year event-free survival rate of more than 80% in TDT patients. However, this study also revealed a 12% overall mortality within 2



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years after allogeneic HSC transplantation and the required (myeloablative) conditioning. In addition, 10% of patients developed severe acute GvHD, and about 15% of patients developed chronic GvHD.[235]

In sickle cell disease (SCD), the β-globin subunit in HbA carries a point variant that results in the formation of an aberrant form termed hemoglobin S. The *HBB* p.Glu6Val variant in combination with the same or a second *HBB* disease-associated variant on the second allele leads to SCD, in which erythrocytes are malformed, resulting in chronic hemolytic anemia. The malformed erythrocytes can cause acute ischemia throughout the body due to obstruction of blood vessels, leading to (severe) pain, organ failure, and severe acute vaso-occlusive complications such as acute chest syndrome or stroke,[236] which can be treated by exchange transfusion.[237] With this therapy, the patients' blood is exchanged with donor blood to lower the percentage of sickle cells. Chronic transfusions are performed in patients with a history of stroke to prevent new cerebral ischemic events.[237] Possible complications of frequent transfusions have been described previously. Frequently hospitalized patients, for example due to acute chest syndrome or the need for intravenous analgesics in the management of acute pain, are treated with hydroxyurea. These treatments, hospital admissions, acute complications, and many more chronic complications result in reduced life quality of patients.[237] As in β-thalassemia, allogeneic HSC transplantation is the only cure for SCD. Although HSC transplantation with a product of a related HLA-matched donor seems successful in most cases, severe complications as described previously are also seen in SCD.[238] Recent improvements in conditioning regimens have led to reduced intensity treatment without short-term GvHD, but serious adverse events still occurred.[239,240] The experience with other HSC transplantation sources is scarce in SCD, but it seems inferior to related HLA-matched donors.[238,241]

Curative options that are less toxic than allogeneic HSC transplantation are required for both β-thalassemia and SCD. As gene therapy allows the engineering of autologous stem cells, the need for a donor would be bypassed. Importantly, transfusion of autologous rather than allogeneic stem cells strongly reduces the HSC transplantation-related toxicity.[242] Reports of gene therapy using lentiviral vectors to add a healthy *HBB* copy to HSCs *in vitro* for reinfusion purposes have been published for β-thalassemia[243] and SCD,[244] and the first promising (interim) results of clinical trials have been reported.[245,246] As the graft must replenish the hematopoietic system through rapid cell division, an integrative vector, such as lentiviral vectors, is required. Although γ-retroviral vectors used in the past gave rise to leukemia through insertional mutagenesis,[247] currently used third-generation self-inactivating lentiviruses have an improved safety profile and have been used without adverse events in several clinical trials up to 7 years follow-up.[248–255] Because lentiviral transduction is highly efficient, it provides a strong competitor for gene editing approaches in strategies involving overexpression of transgenes.

The main strategy under current investigation for clinical application of gene editing is the induction of endogenous expression of fetal hemoglobin (HbF). This originated from the observation that co-inheritance of hereditary persistence of HbF (HPFH), a benign condition, reduces symptoms of SCD and β-thalassemia.[227] The situation in SCD and β-thalassemia is depicted in Figure 3A. In HPFH, HbF protein production continues into adulthood, whereas under normal physiological conditions production shifts to adult hemoglobin after birth. HbF protein contains two subunits of α-globin and γ-globin each, the latter of which are translated from the *HBG* gene. Persistent HbF expression in HPFH compensates for the reduced production of HbA in β-thalassemia patients. There is a difference in HbF protein levels among β-thalassemia patients, and this has been linked to several genetic variants, with single nucleotide variants (SNVs) in the *BCL11A* gene correlating most strongly with HbF expression.[256] Reduced BCL11A protein expression is correlated with increased HbF protein expression, likely because BCL11A suppresses HbF expression by binding directly to the *HBG* promoter.[257,258] *BCL11A* null mice were shown to be unable to downregulate murine embryonic globin in erythrocytes, demonstrating the essential role of BCL11A in repression of HbF expression during development.[259] However, BCL11A knockdown by gene disruption in HSCs results in impaired engraftment of HSC in mice, illustrating that knockout of BCL11A itself is not a feasible strategy to treat β-thalassemia.[260] As BCL11A expression during erythropoiesis is specifically regulated by the intronic erythroid-specific enhancer,[261] disrupting this enhancer will result in BCL11A knockout during erythropoiesis, exclusively. This strategy was preclinically tested by using ZFN-mediated gene disruption of the GATAA element of the BCL11A erythroid-specific enhancer in HSCs (Figure 3B).[260,262] These cells achieved robust long-term engraftment in mice and gave rise to erythroid cells with elevated HbF levels upon *ex vivo* culture of chimeric bone marrow.[260] Multiple clinical trials are based on a

Figure 3. Gene Editing Strategies in β-Thalassemia and Sickle Cell Disease

(A) Situation in β-thalassemia and sickle cell disease. The locus control region (LCR) loops to the β-globin gene and β-globin is expressed; however, due to a disease-associated variant in the β-globin gene there is insufficient expression (β-thalassemia) or malformed (sickle cell disease) β-globin. The transcriptional repressor BCL11A recognizes the first TGACCA binding sequence, which leads to inhibition of expression of fetal-specific γ-globin. (B) In one strategy, CRISPR-Cas9 or ZFNs (not shown) are used for targeted disruption of the GATAA motif in the intronic erythroid-specific enhancer of *BCL11A*, which will result in disruption of *BCL11A* expression during erythropoiesis and consequently relief of inhibition of γ-globin expression. γ-Globin will substitute for the lack of β-globin to form functional hemoglobin: HbF. (C) In a related strategy, the TGACCA recognition site for BCL11A is disrupted using CRISPR-Cas9 or ZFNs (not shown). BLC11A remains expressed but cannot bind to the recognition site to inhibit the γ-globin expression, resulting in relief of inhibition of γ-globin expression. (D) In another scenario, the β-globin promotor sequence is disrupted using CRISPR-Cas9, leading to a loss of binding sites for proteins that repress expression of γ-globin and subsequent induction of γ-globin expression. (E) Finally, the disease-associated variant can be precisely corrected using CRISPR-Cas9. While strategies in (B), (C), and (D) will lead to the induction of fetal hemoglobin, the strategy in (E) will lead to production of adult hemoglobin. Red indicates the result of intervention.

strategy involving HSCs, of which the intronic erythroid-specific enhancer of BCL11A is disrupted *ex vivo* using CRISPR-Cas9 or ZFNs as a treatment for TDT[263,264] or SCD[265,266] patients. The long-term effects of infusing such cells are investigated in one clinical trial.[267] Other strategies to increase HbF expression include disruption of the binding motif for BCL11A (and co-repressive proteins) within the HBG promoter sequence (Figure 3C)[257,268,269] or the induction of a natural occurring variant termed the Sicilian HPFH disease-associated variant (Figure 3D).[270] In the latter variant, the entire β-globin locus is deleted and the putative 3′ β-globin enhancer is brought in closer proximity to the γ-globin locus. These strategies have been explored preclinically, but have not (yet) reached clinical application.

Besides induction of HbF, other applications of gene editing techniques to treat β-thalassemia and SCD have been tested mainly in preclinical studies. Cai et al.[271] showed an approach to correct various *HBB* disease-associated variants by inserting a cDNA sequence of exons 2 and 3 of the *HBB* gene downstream of *HBB* exon 1 *in vitro* using CRISPR-Cas9 in induced pluripotent stem cells (iPSCs). This strategy ensured expression of correct β-globin and prevented expression of the mutated variant in iPSC-derived erythrocytes. Other preclinical studies showed the (HDR-mediated) correction of a specific disease-associated variant in (iPSC-induced) HSCs to restore β-globin and thus HbA expression (Figure 3E).[272–279] One clinical trial implies to investigate the infusion of autologous, iPSC-induced HSCs with a directly gene-corrected version of the *HBB* gene in β-thalassemia.[280] However, very limited information is provided for this trial and the exact strategy is not elucidated. Another preclinical strategy involves the *in vitro* knockout of α-globin[281] to prevent its precipitation. No clinical trial has been reported that investigates this option.

Gene Editing in Hemophilia

Hemophilia A and B are congenital bleeding disorders caused by deficiencies in clotting factor VIII (FVIII) or IX (FIX), respectively. These diseases have a recessive X-linked inheritance pattern. Protein substitution therapy (PST) with recombinant clotting factor or protein derived from donor plasma is currently the main treatment for these patients.[282] Despite the steep increase in life expectancy and the improved prevention of arthropathies due to articular bleedings after introduction of PST, this treatment has its drawbacks.[282,283] As substituting a deficient protein is not curative, repeated administration is required and patients remain at risk of bleedings. In addition, costs related to PST are considerable.[282] Insertion of a functional copy of the deficient gene in patient cells could potentially provide a long-term cure for hemophilia. To this end, *in vivo* gene therapy by viral vectors has been applied in multiple phase I clinical trials,[284–286] as well as by *ex vivo* electroporation of fibroblasts that provided a source of FVIII after engraftment.[287] Initial results observed in these clinical trials were disappointing.[282] For lentiviral transduction, preclinical optimization of *ex vivo* HSC-mediated lentiviral gene therapy is paving the way for the first clinical studies.[282] In spite of subclinical effects of targeting muscle cells by AAV vectors in hemophilia, promising clinical results have been obtained by the use of AAV vectors targeting liver cells.[282] Transient liver toxicity and a temporary requirement for immunosuppressive therapy were drawbacks of this strategy.

Currently, gene editing strategies to target liver cells are also being explored for hemophilia. Sharma et al.[288] achieved robust expression of human FVIII or FIX by integrating the cDNA of either gene into intron 1 of the albumin locus in primary hepatocytes *in vitro* and in hepatocytes of mice *in vivo* by using AAV-delivered ZFN-mediated gene editing. Despite the low *in vivo* genome editing efficiency, gene expression was achieved by placing the genes under the control of the highly active albumin promoter. This *in vivo* gene editing strategy is currently being investigated in hemophilia B patients in a clinical trial.[289] A drawback for clinical implementation of such strategy is the long-term presence of active gene editing components in the liver of patients and the associated risk of damaging the genome by introducing double-stranded breaks at off-target loci. This is a serious concern, as the gene editing machinery delivered by AAV has an expected presence in the liver of several years, which significantly increases the chance for off-target effects to occur. This highlights the need for developing more transient ways to perform *in vivo* gene editing.

Other preclinical strategies that are under investigation include insertion of the transgene into the AAVS1 locus[290,291] or in the native locus[292–295] and correction of disease-associated variants[296–298] or large chromosomal rearrangements.[299–301]

For the clinical translation of gene editing in HSCs, a critical aspect is to maintain long-term engraftment capacity.[201,302–305] Similar to most other cells, HDR-mediated gene editing is challenging in HSCs, as these cells prefer the NHEJ pathway. In addition, it has been found that genetic manipulation of HSCs with gene editing or viral vectors can reduce their engraftment capacity. This has been found to be caused by activation of the DNA damage response pathway, resulting in activation of p53. Transient inhibition of p53 has been found to improve long-term engraftment of HSCs after gene editing.[344] In addition, technical optimizations related to cell culture, delivery, and use of reagents have resulted in enhanced long-term engraftment of HSCs after gene editing in xenograft experiments involving transplantation of human HSCs into immunodeficient mice. The clinical testing of long-term engraftment of gene-edited HSCs in human patients needs further testing.

Gene Editing in Metabolic Disorders

Mucopolysaccharidoses

Mucopolysaccharidoses (MPSs) are monogenic lysosomal storage diseases (LSDs) in which one of the enzymes involved in the lysosomal degradation of glycosaminoglycans (GAGs) is deficient. In MPS I and II, this concerns the α-L-iduronidase (IDUA) and iduronate-2-sulfatase (IDS) enzymes, respectively. Patients suffer from multisystemic symptoms and reduced life expectancy that can vary depending on the type of MPS and the severity of the

disease-associated variant.[306] The currently available treatment for MPS I, MPS II, MPS IVA, MPS VI, and MPS VII is enzyme replacement therapy (ERT), in which recombinant enzyme is administered intravenously. Drawbacks of ERT include the non-curative nature of the treatment, the requirement of repeated intravenous infusions, high costs, and ineffectiveness in treating symptoms in bone, cartilage, heart valves, and the central nervous system.[307,308] In addition, generation of antibodies against the recombinant enzyme can interfere with the efficacy of ERT.[307] HSC transplantation is currently applied to treat MPS I.[309] This relies on the principle that lysosomal enzymes are secreted and can be taken up by target cells via the cation-independent mannose 6-phosphate receptor (CI-M6PR). In HSC transplantation, HSCs and their progeny secrete the enzyme into the circulation and provide a continuous source of ERT. In the case of MPS I, the level of secretion and reuptake provides partial efficacy in target organs. However, HSC transplantation depends on the availability of HLA-matched donors and can have severe adverse events such as GvHD, infection, and even death, as described before.[306] In addition, the therapeutic effect on the skeletal abnormalities and neurological symptoms is limited, and for many other LSDs, endogenous expression levels in HSCs are insufficient to treat target organs. Therefore, overexpression by *ex vivo* lentiviral transduction or gene editing provides (additional) therapeutic efficacy. For MPS I, liposome-mediated delivery of CRISPR-Cas9 has been successfully applied *in vivo* and resulted in increased IDUA expression in newborn MPS I mice.[310] Alternatively, direct gene addition using AAV vectors (without gene editing) has been shown feasible in preclinical studies for several MPS types.[311–316] This strategy is being investigated in multiple clinical trials, and recent results using intracerebral delivery showed promising outcomes with respect to treating the neurological decline of MPS IIIB patients.[317]

Another approach, similar to the approach in hemophilia, is the site-specific integration of a transgene in the liver by *in vivo* genome editing following intravenous administration using AAV as the delivery method.[288] Most efforts have been made on integrating transgenes into the albumin locus. Sharma et al.[288] achieved ZFN-mediated insertion of IDUA and IDS *in vivo* into the albumin locus of healthy mice, resulting in detectable protein levels in liver lysates. More recently, ZFN-mediated insertion of human IDS in the albumin locus in murine liver *in vivo* was accompanied by a dose-dependent rise in circulating enzyme levels.[318] This IDS insertion caused reduction of GAG levels in tissue and urine samples of MPS II mice. These results have led to clinical trials investigating the safety of ascending dose levels of AAV vectors containing components required for *in vivo* ZFN-mediated insertion of IDUA and IDS genes into the albumin locus of hepatocytes in the liver of MPS I patients[319] and MPS II patients,[320] respectively. The same drawbacks as in the hemophilia trial with respect to safety due to the potential introduction of double-stranded breaks in the liver at off-target locations in the genome apply here due to the long-term exposure of the patient to the uncontrolled activity of ZNF-mediated double-stranded breaks.

Gene Editing in the Eye

Leber's Congenital Amaurosis

Leber's congenital amaurosis (LCA) is an inherited retinopathy in which severe visual impairment or blindness occurs within the first months of life.[321] It is a genetically heterogeneous disease that can be caused by any of more than 20 mutated genes. Based on the genes involved and the ocular phenotypes, LCA is divided into 13 subtypes.[322] Currently, there is no treatment for LCA. In clinical trials, it has already been shown that AAV-mediated gene transfer by subretinal injection resulted in improved visual parameters in patients with the LCA type LCA2, which is caused by variants in the *RPE6* gene.[323–326] Retinal dystrophy in LCA was (at least partially) reversed by the therapy. AAV-mediated gene therapy has also been applied to other congenital retinopathies.[327]

In addition to AAV-mediated gene transfer, gene editing is in development for retinopathies. For subtype LCA10, which is caused by variants in the *CEP290* gene,[321,328] a clinical trial is currently open[329] with the strategy outline below. Gene transfer via viral vectors (especially AAV) is problematic for *CEP290* due to the large gene size. *CEP290* encodes a protein that is essential for cilia, which are microtubule-based, hair-like extensions of cell membranes.[330] In photoreceptor cells, cilia are highly specialized into cone- or rod-shaped segments that act as light sensors and signal transducers.[322] In LCA10, *CEP920* disease-associated variants cause (peripheral) thickening of the retina by an unknown mechanism.[330] The most common variant is the intronic variant IVS26, which results in the generation of a cryptic splice site that causes an abrogated protein product.[328] Preclinical studies had shown that, using subretinal injections of AAV5 vectors containing the CRISPR-Cas9 gene editing machinery, it deletion of the cryptic splice site leads to restoration of canonical splicing and expression of wild-type protein.[331,332] This concept is used in the ongoing clinical trial.[329] Other preclinical studies are investigating gene editing strategies for other disease-associated variants in LCA and other retinopathies.[333,334] However, long-term expression of CRISPR-Cas9 in the eye imposes safety risks, as discussed in approaches for *in vivo* gene editing in hemophilia and MPS I and II.

Conclusions and Future Prospects

Disease-Specific Challenges

The challenges and opportunities of applying gene editing for the treatment of human disease depend in part on disease-specific aspects. In cancer immunotherapy, a major challenge is to specifically target cancer cells while leaving healthy cells unharmed. Targeting immune checkpoints with gene editing has been shown to be a promising strategy, but the clinical feasibility relies in part on the inherent problem of specificity: by inhibiting a general checkpoint with the aim to inhibit negative immune regulation, there is a risk of auto-immune-related side effects. Considering the life-threatening nature of cancer, this disadvantage may be acceptable if survival rates can be improved and increased toxicity is manageable. Other challenges include the viability of T cells that have been gene edited *ex vivo* to knock out immune checkpoint regulators. These cells do not need

to be present life-long, but they should have sufficient viability in order to help eliminating cancer cells. If needed, repeated administration would be an option, but this will increase costs. The development of a universal ACT would be an elegant solution to the high costs of preparing autologous or HLA-matched allogeneic gene-edited T cells for each individual patient, although this approach has the risk of inducing GvHD.

Application of targeted knockout to viral infection such as HPV could provide a useful additional treatment option when it comes to treating the primary tumor. However, a high effciency of gene knockout is reqired to effectively reduce the tumor, and treating metastases is not yet possible due to the difficulty to reach target tissues and to eliminate the HPV virus in a safe and efficient manner outside the primary tumor. It might be an advantage to target viral sequences rather than endogenous genomic locations to reduce the risks of undesired genomic alterations as the result of gene editing. This could also be a potential advantage for strategies that eradicate HIV provirus from the genome. In the case of HIV, disease-specific challenges include the targeting of the dormant HIV reservoir, and to target HIV strains that do not depend on CCR5 for infection.

Ex Vivo *Gene Editing*

In both genetic disease and viral infection, promising strategies using *ex vivo* gene editing lie ahead for disorders that can be cured via the blood, including hematological disorders, lysosomal storage disorders, and HIV infection. The main reason for this is the feasibility to target blood cells such as HSCs or T cells *ex vivo* and to engraft autologous gene-modified cells back into patients. This approach relies on the long-standing experience with successful engraftment of HSCs, which has nowadays become a standard procedure with a very good safety profile. In addition, engrafted HSCs can provide a life-long treatment because they can self-renew to sustain the stem cell population and to differentiate into the hematopoietic lineage. Because prolonged *ex vivo* culture reduces engraftment potential and stem cell properties of HSCs, fast and efficient methods are required to make *ex vivo* gene editing of HSCs feasible for clinical implementation. It remains to be seen whether *ex vivo* gene editing for overexpressing proteins will be able to successfully compete with *ex vivo* lentiviral transduction of HSCs when it comes to clinical implementation, because lentiviral transduction is highly efficient, has been used more than 7 years without adverse events in several clinical trials, and could be more cost-effective.[248–255] Strategies that rely on NHEJ are inherently more efficient compared to the HDR-mediated insertion of transgenes, and these provide promising options for the treatment of HIV infection, by knocking out the CCR5 receptor, or some genetic disorders such as β-thalassemia and SCD, by knocking out regulatory elements required for BCL11A-mediated negative regulation of HbF expression.

Among the many other preclinical developments for using *ex vivo* gene-edited HSCs (not covered in this review), the primary immunodeficiency diseases (PIDs) represent a promising example.[335,336] These patients usually benefit from allogeneic HSC transplantation from HLA-matched donors, but these are not always available, and allogeneic HSCs can induce GvHD. Autologous HSC transplantation following *ex vivo* gene therapy employing third-generation lentiviruses is ongoing in a number of clinical trials for Wiskott-Aldrich syndrome, ADA severe combined immunodeficiency (ADA-SCID), X-linked SCID, and chronic granulomatous disease (CGD). However, many PIDs involve genes with a timed and restricted expression pattern during development and require endogenous expression levels via the natural promoter rather than overexpression. Gene editing would be advantageous above lentiviral transduction in these cases, as it enables precise correction of an endogenous allele to maintain endogenous expression levels. There are currently no clinical trials for PIDs reported using gene editing, but promising preclinical developments may change this in the near future.

Other promising preclinical developments include the *ex vivo* gene editing of primary hepatocytes for metabolic disease of the liver. As a proof of concept, AAV-mediated delivery of CRISPR-Cas9 to freshly isolated mouse hepatocytes was used, followed by engraftment into the liver of a mouse model. This concept was applied to treat hereditary tyrosinemia in a mouse model to correct a point variant in the fumarylacetoacetate hydrolase gene using HDR.[337] A major challenge for this approach is the limited engraftment capacity of hepatocytes in human liver. In cystic fibrosis, investigators are pursuing gene editing of stem cells derived from the airways with the ultimate goal of developing a gene-edited autologous airway stem cell transplantation.[338] Mitochondrial diseases that are caused by disease-associated variants in mitochondrial DNA form an attractive target for gene editing.[339,340] However, gene editing of mitochondrial DNA is even more challenging than nuclear DNA, and improvements are required before clinical applications can be considered in the near future.

In all of these possible applications, the *ex vivo* mode of gene editing ensures that a quality control can be performed prior to decision-making of engrafting cells into patients. Reliable methods to assess undesired genomic alterations are essential, and a shift from methods that rely on predictions toward unbiased methods will be required. Quality control should also include functional analysis of cellular transformation, because rare events that result in formation of tumorigenic cells will be very difficult to detect in population-based assays.

In Vivo *Gene Editing*

In vivo gene editing trials have already started for a number of disorders including lysosomal storage disorders, hemophilia, precancerous cervical lesions and LCA. This is despite the uncertainties of gene editing technology with respect to possible off-target effects. This is particularly important when gene editing technology is introduced in patients without ways for spatiotemporal control (i.e., means to confine gene editing to a short time and specific target tissue, for example by using suicide genes in DNA combined with tissue-specific delivery, or local administration of gene editing tools as RNA/protein rather than DNA), such as is the case in trials so far. This means that gene editing may continue for years inside the patient, which will increase the risk of undesired events with several orders of magnitude

compared to *ex vivo* gene editing. For safe future clinical development, it will be important to develop ways that can control the activity of *in vivo* gene editing by including on and off switches to prevent the prolonged generation of DNA breaks or by providing the gene editing machinery in other ways than as DNA. In addition, targeting gene editing tools specifically to the cells of interest will further increase the safety by preventing unnecessary targeting events in irrelevant cell types.

These aspects will also guide ongoing preclinical efforts to develop treatments for human disease based on *in vivo* gene editing. Multiple preclinical developments in different fields are ongoing, and it is beyond the scope of this review to cover these. As examples we mention metabolic disorders that are amenable to correction via knockout of a gene in the metabolic pathway to enable redirecting of metabolism. For example, severe hereditary tyrosinemia type I was successfully redirected to a more begin tyrosinemia type III form by deletion of the upstream metabolic enzyme hydroxyphenylpyruvate dioxygenase in the liver. The method applied involved intravenous injection of DNA encoding Cas9 and sgRNAs in the mouse, which transfected the liver more efficiently compared to other organs.[341] The same gene was also targeted in as study on *in utero* correction of hereditary tyrosinemia type I using injection of an adenovirus expressing a base editor and sgRNA into mouse fetuses via the vitelline vein. In the same study, in utero knockout of PCSK9 was achieved with the aim to lower cholesterol levels and the risk of coronary heart disease in wild-type mice.

Precise correction of a point variant *in vivo* has been demonstrated for example in a mouse model for phenylketonuria (PKU) using base editors that were delivered by intravenous injection and that were expressed via a liver-specific promoter.[342] Gene editing is even applied in preclinical research to increase the fitness of pig organs for future xenotransplantation into humans. By knockout of genes that activate an immune response and retroviral elements, the aims are to generate organs with reduced hazard of graft rejection and xenozoonosis (an infectious disease transmitted from animal to human), respectively.[343] The ultimate goal of these efforts is to overcome the shortage of human organs such as kidneys, hearts, livers, and lungs for transplantation.

Keeping Up with Technological Developments

Finally, it will be important to educate the various stakeholders, including clinicians, patients, and regulatory institutions. The technology for gene editing is moving so fast that it is difficult to cope with all of the developments and their potential benefits and risks. Clinicians need to be educated in order to allow them to judge the feasibility of a clinical trial and whether they are willing to expose their patients to the novel treatment. Patients rely largely on the information that is provided by their treating physician. The prospect of a "cure" via gene repair may be tempting for a patient, and therefore providing balanced and fair information by the physician on the possible benefits and risks provides an essential ingredient for decision-making. The same arguments apply to regulatory institutions, as these will approve or decline clinical protocols and finally market authorization. While the scientific developments in the field of gene editing are continuing with dazzling speed, it will be important to provide education in the field and to closely monitor and regulate clinical developments.

In this review, we compiled all current clinical applications of gene editing and explained the rationale for the underlying strategies. In addition, we summarized preclinical studies that preceded clinical trials and provided examples of preclinical work that might be translated in a clinical setting in the future. As most other reviews focus on specific areas involving gene editing applications, we envision that centralized information on gene therapies will increase awareness of clinicians and researchers in the field of gene therapy outside their specific field of interest, and that this might catalyze new developments. We propose that clinical applications of gene editing in general will be documented in an accessible and transparent manner. We hope that this review precedes the discussion of a central database that includes relevant information of the clinical studies applying gene editing, as well as the underlying considerations with respect to the mechanism of action, safety, and expected results. Ideally, this information should be contributed by investigators involved in these clinical trials, peer-reviewed by experts in the field, and made publicly available prior to the start of such trials. Preferably, an analysis of risks and benefits of gene editing for a specific disease in the context of current treatments should be included, contributing to discussions on technical and ethical aspects of the applications. Such efforts should contribute to increasing transparency and help to inform stakeholders that are involved in clinical trials involving gene editing.

AUTHOR CONTRIBUTIONS

M.P.T.E., P.H.-H., M.B., and W.W.W.P.P. conceptualized this review, performed literature studies, and wrote the manuscript. All authors interpreted the contents and approved the final manuscript.

CONFLICTS OF INTEREST

A.T.v.d.P. has provided consulting services for various industries in the field of Pompe disease under an agreement between these industries and Erasmus MC, Rotterdam, the Netherlands. The remaining authors declare no competing interests.

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Chapter 4

Large-Scale Expansion of Human iPSC-Derived Skeletal Muscle Cells for Disease Modeling and Cell-Based Therapeutic Strategies

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Large-Scale Expansion of Human iPSC-Derived Skeletal Muscle Cells for Disease Modeling and Cell-Based Therapeutic Strategies

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SUMMARY

Although skeletal muscle cells can be generated from human induced pluripotent stem cells (iPSCs), transgene-free protocols include only limited options for their purification and expansion. In this study, we found that fluorescence-activated cell sorting-purified myogenic progenitors generated from healthy controls and Pompe disease iPSCs can be robustly expanded as much as 5×10^{11}-fold. At all steps during expansion, cells could be cryopreserved or differentiated into myotubes with a high fusion index. *In vitro*, cells were amenable to maturation into striated and contractile myofibers. Insertion of *acid α-glucosidase* cDNA into the *AAVS1* locus in iPSCs using CRISPR/Cas9 prevented glycogen accumulation in myotubes generated from a patient with classic infantile Pompe disease. *In vivo*, the expression of human-specific nuclear and sarcolemmar antigens indicated that myogenic progenitors engraft into murine muscle to form human myofibers. This protocol is useful for modeling of skeletal muscle disorders and for using patient-derived, gene-corrected cells to develop cell-based strategies.

INTRODUCTION

Although over 700 human genetic disorders are known that affect skeletal muscle (Kaplan and Hamroun, 2015), very few therapies are available. Skeletal muscle nonetheless has a high capacity for regeneration after injury (Baghdadi and Tajbakhsh, 2017; Bursac et al., 2015; Dumont et al., 2015). Muscle regeneration is mediated by satellite cells (SCs) (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011); i.e., adult stem cells located between the sarcolemma and the plasma membrane (Mauro, 1961) that are quiescent in healthy, uninjured muscle. Upon injury, SCs expand to contribute to fiber formation and to self-renew the SC pool.

SCs are considered useful for *in vitro* disease modeling to investigate molecular mechanisms of disease, test drugs, or develop cell-based therapies. To decipher molecular mechanisms of disease, it is important to generate isogenic controls, given the high variability of gene expression and functional parameters between individuals (Hockemeyer and Jaenisch, 2016; Soldner et al., 2011). To develop cell-based therapy, the ultimate goal is to engraft gene-corrected, autologous cells. However, it has not proved easy to date to establish robust *in vitro* disease models for skeletal muscle disorders, to efficiently restore gene function in skeletal muscle cells, and to develop cell-based therapeutic strategies based on muscle regeneration.

Pluripotent stem cells (PSCs) offer a potential source of skeletal muscle cells. PSCs, including induced PSCs (iPSCs), are easily expanded and maintain their full stem cell potential (Takahashi and Yamanaka, 2016). Differentiation of PSCs to SC-like cells was difficult until the recent development of two major strategies, the first involving the inducible overexpression of PAX7, the master transcription factor for SCs (Darabi et al., 2012). After generation from human embryonic stem cells and iPSCs, purified SC-like cells showed capacity for *in vitro* expansion and differentiation, and also for *in vivo* engraftment and contribution to muscle-fiber formation in immunodeficient mice (Darabi et al., 2012; Magli et al., 2017). The second strategy involved the use of small molecules to develop transgene-free differentiation. After using GSK3β inhibition to activate the Wnt pathway, the basic procedure consists of treatment with fibroblast growth factor 2 (FGF2) and culturing in a minimal medium (see Table S1) (Borchin et al., 2013; Caron et al., 2016; Shelton et al., 2014, 2016; van der Wal et al., 2017b; Xu et al., 2013). In some cases, differentiation into the myogenic lineage has been promoted by including BMP4 inhibition (Chal et al., 2015, 2016; Swartz et al., 2016). In others, FGF2 has been replaced by the Notch signaling inhibitor DAPT (Choi et al., 2016).

Transgene-free protocols can be divided into those that use fluorescence-activated cell sorting (FACS) purification (Borchin et al., 2013; Choi et al., 2016; van der Wal et al.,

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2017b) and those that use unpurified cell mixtures or partial purification through preplating (Caron et al., 2016; Chal et al., 2015; Shelton et al., 2014; Swartz et al., 2016; Xu et al., 2013) (Table S1). Upon terminal differentiation *in vitro*, unpurified/partially purified myogenic progenitors showed matured myotubes and even myofibers (Chal et al., 2015, 2016; Swartz et al., 2016). Three reports showed engraftment of myogenic cells from unpurified cultures into immunodeficient mice (Choi et al., 2016; Kim et al., 2017; Xu et al., 2013). Choi et al. (2016) reported that purification of myogenic progenitors by FACS resulted in myogenic progenitors that could be expanded 10^5-fold. Upon *in vitro* differentiation to myotubes, these cells also showed a low (10%–15%) fusion index (Table S1).

In vivo engraftment of purified myogenic progenitors using a transgene-free procedure has not been reported so far. Similarly, it has not been possible yet to expand transgene-free, purified myogenic progenitors and differentiate and mature these cells to myotubes with high fusion index. Recently, we have modified a protocol by Borchin et al. (2013) for the transgene-free differentiation of human iPSC into SC-like cells, and used a simplified FACS purification procedure that selects C-MET-expressing cells that are HNK negative (Borchin et al., 2013; van der Wal et al., 2017b). The purified cells could be expanded at least 5×10^7-fold and cryopreserved. At any point during the expansion, cells could be differentiated into myotubes with a high (60%–80%) fusion index. We have applied this protocol to model Pompe disease, which is a progressive inheritable metabolic myopathy caused by deficiency of acid α-glucosidase (*GAA*), resulting in lysosomal glycogen accumulation (van der Ploeg and Reuser, 2008). This protocol allowed the quantitative analysis of the effects of antisense oligonucleotides designed to restore canonical pre-mRNA splicing of *GAA* in skeletal muscle cells from Pompe patients (van der Wal et al., 2017a).

Here, we further explored the expansion capacity and the *in vitro* and *in vivo* potential of myogenic progenitors, generated from iPSCs in a transgene-free manner and FACS purified, for the future development of therapies for skeletal muscle disorders.

RESULTS

Optimization of the Generation of Myogenic Progenitors from iPSCs

As a starting point, we took the protocol published by Borchin et al. (2013), which we had modified recently (van der Wal et al., 2017b). This protocol consists of treating human iPSCs first with the GSK3β inhibitor CHIR99021, then with FGF2, followed by prolonged culturing in minimal medium. The treatment with CHIR99021 is a critical step, as too-low concentrations fail to yield myogenic progenitors, while too-high concentrations can be toxic. The optimal concentration most likely depends on the cell culture conditions used. We assume, for example, that the outcome can be affected by culturing iPSCs with or without feeders.

In our experiments, we cultured iPSCs on γ-irradiated mouse embryonic fibroblasts. To determine the optimal treatment with CHIR99021, we varied the concentration and duration of treatment and scored for confluency and PAX7 expression (Table S2). The results in two independent iPSC lines showed that the highest number of $PAX7^+$ cells was induced after 4–5 days at a concentration of 4 μM CHIR99021 in the absence of toxicity. To avoid any risk of toxicity in subsequent experiments, we chose 5-day incubation at a concentration of 3.5 μM CHIR99021.

Robustness of the Myogenic Differentiation Protocol

As outlined in Figure 1A, we used primary fibroblast-derived iPSCs from 15 different donors, applying the myogenic differentiation procedure in over 50 individual differentiation experiments. Eight of these iPSC lines were derived from healthy individuals, while seven were from patients with Pompe disease. Figure 1B shows robust generation of $PAX7^+$ areas in six examples of healthy control iPSCs after 35 days of differentiation as described previously (van der Wal et al., 2017b). During the differentiation procedure, phase-contrast microscopy showed small colonies with a confluency of between 20% and 40% at day 1 (Figure S1A). After 5 days of culture, iPSC colonies had reached a medium size. At this stage CHIR99021 treatment was started. After 5 days of incubation, we observed increased cell detachment, which was attenuated after a further 3–4 days in FGF2-containing medium. From day 17 onwards, the cells started to proliferate rapidly, and cultures reached complete confluency after 24 days. Multinucleated myotube-like cells were observed between 30 and 40 days. During this differentiation procedure, we observed similar morphological changes in all iPSC lines (Figure S1A and data not shown).

Differentiation of 59 cultures from a total of 15 donors yielded an average of 4.26% ± 3.96% of $C\text{-}MET^+/Hoechst^+/HNK\text{-}1^-$ cells (Figure 1C). There were no significant differences in the number of $C\text{-}MET^+/Hoechst^+$ cells between iPSCs from healthy controls and from Pompe patients. Sorting differentiation cultures with low levels of $C\text{-}MET^+/Hoechst^+/HNK\text{-}1^-$ cells (~0.2% of cells) resulted in expandable myogenic progenitors whose differentiation capacity was similar to that of cultures with a high recovery (>2%) (data not shown). C-MET-/$HNK\text{-}1^+$ cells were unable to form myosin heavy chain (MHC)-positive cells after 4 days of differentiation (data not shown). After 24 hr of plating, sorted myogenic progenitors revealed a rather

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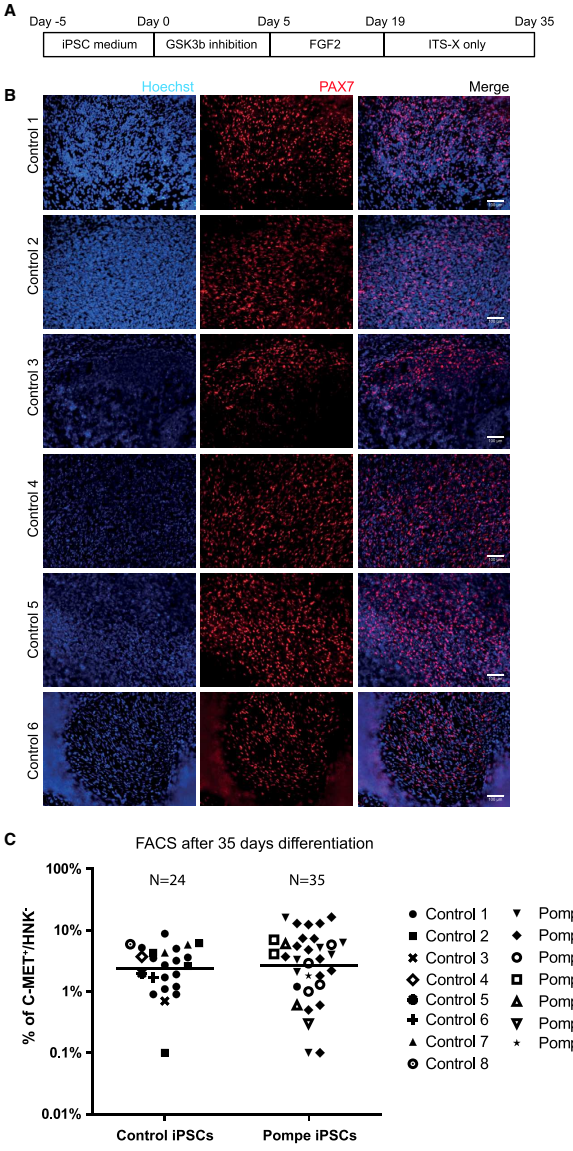

Figure 1. Robustness of Generation and Purification of Myogenic Progenitors from iPSCs

(A) Scheme for myogenic differentiation of iPSCs.

(B) Representative examples of PAX7$^+$ cells obtained in the original culture dishes from six different control iPSC lines that were differentiated using a 35-day protocol consisting of consecutive treatment with CHIR, FGF2, and minimal medium (Borchin et al., 2013; van der Wal et al., 2017b). The other two control iPSC lines showed similar patches of PAX7$^+$ cells (data not shown). Red: PAX7$^+$ nuclei using immunofluorescent staining. Blue: nuclei stained with Hoechst. For images of the plates during this 35-day protocol see Figure S1A.

(C) Differentiations described in (B) were purified using a one-step FACS purification based on selection for C-MET$^+$ myogenic cells and counter selection of HNK1$^+$ neural crest cells (Borchin et al., 2013). Results are shown for 59 differentiations performed on iPSCs derived from eight healthy controls and seven Pompe patients. Each symbol represents an individual differentiation experiment. Means are indicated by horizontal lines. A total number of 24 differentiations of control iPSCs and 35 differentiations of Pompe iPSCs were performed.

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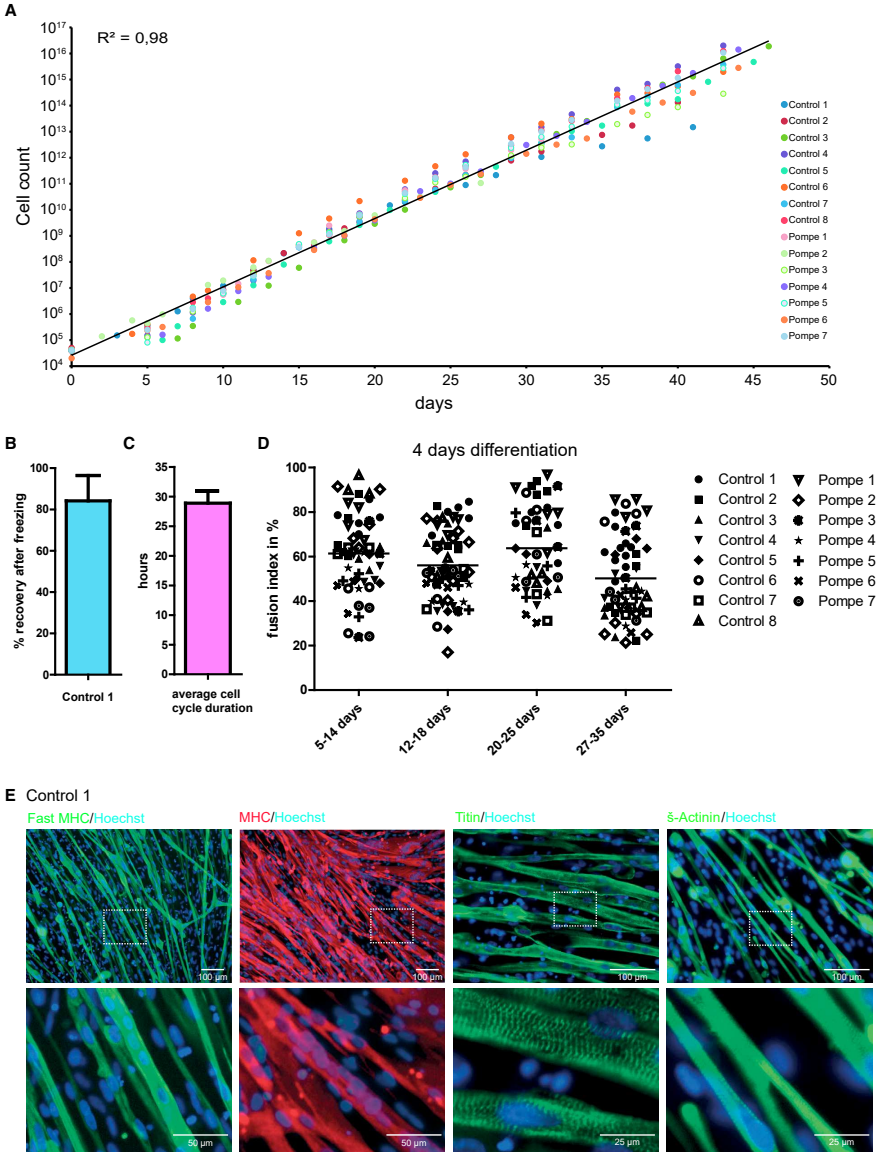

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uniform morphology (Figure S1B). These results demonstrated that the differentiation protocol robustly generated C-MET$^+$/Hoechst$^+$/HNK$^-$ myogenic cells.

In Vitro Expansion, Differentiation, and Maturation of Purified Myogenic Progenitors

During 31 days of culture we had previously determined the proliferation rate of purified myogenic progenitors derived from two healthy controls and two Pompe patients (van der Wal et al., 2017b). To further determine expansion capacity, we determined the expansion capacities of myogenic progenitors generated from iPSCs from six additional healthy controls and five additional patients with Pompe disease. Proliferation rates were observed for all myogenic progenitor lines that reached 2×10^{16} cells during 43 days of expansion (Figure 2A). Cells could be cryopreserved with 84% recovery (Figure 2B) without affecting differentiation capacity (data not shown), and showed an average cell cycle of 28.9 hr (Figure 2C). After 43 days of expansion, the proliferation rate diminished, the morphology of cells changed, and differentiation capacity decreased (data not shown). This showed that the myogenic progenitors generated with this protocol could be expanded by a maximum of 5×10^{11}-fold.

Previously we had used a 4-day differentiation protocol to demonstrate that the differentiation capacity remained intact during the expansion phase of myogenic progenitors, based on similar fusion indexes (van der Wal et al., 2017b). Here we extended this analysis to demonstrate that all myogenic progenitors derived from eight healthy control and seven Pompe iPSCs retain their capacity to differentiate into multinucleated myotubes during expansion (Figure 2D). The average fusion index ranged between 20% and 97% and showed no expansion-induced differences (Figure S2A). Next, we tested whether maturation to contractile skeletal muscle cells is possible from purified myogenic progenitors. However, extending culture of myogenic progenitor-derived myotubes in conventional differentiation medium (1% ITS-X [insulin-transferrin-selenium-ethanolamine] in DMEM/F12) beyond day 4 of differentiation increased cell detachment and death (data not shown). Supplementation of the myogenic progenitors' differentiation medium with 0.5%–2% fetal bovine serum increased the overall survival of the culture but also increased the proliferation rate of mononucleated cells, resulting in overgrowth of the cell culture (data not shown). In contrast, supplementation with 1% knockout serum replacement supported further differentiation of myogenic progenitors into skeletal muscle cells for up to 12 days. Longer differentiation resulted in fibers that expressed fast MHC, MHC, titin, and α-actinin; that showed patterns of striation (Figures 2E and S2B); and that contracted spontaneously (Videos S1 and S2). This demonstrated that functional sarcomeres, the strongest evidence of terminal differentiation, were formed.

Generation of Gene-Corrected Myogenic Progenitors Using CRISPR/Cas9-Mediated Insertion of a cDNA into a Safe Harbor

Using gene editing, it is possible to perform genetic correction of human disease *in vitro* by placing an extra copy of the wild-type gene into a so-called safe harbor; i.e., a safe location of the genome (Hockemeyer and Jaenisch, 2016). As such a strategy relies on homology-directed DNA repair, which is inefficient, we generated a targeting construct that allows the selection and subsequent removal of the selection marker. The generic donor vector is shown in Figure 3A. As a proof of concept, we chose the PPP1R12C gene in the AAVS1 locus (Figure 3B) (Lombardo et al., 2011). As well as unique restriction sites that enable cloning of the 5′ and 3′ homology arms, the donor vector contains a ubiquitous EF1α promoter in front of the cDNA of interest (flanked by unique restriction sites); a poly(A) site; and a *neomycin* selection marker driven by the CAG promoter flanked by loxP sites, which provide the option of removing the selection marker by transient expression of CRE recombinase (Figure 3A).

As proof of principle, we aimed to correct the glycogen accumulation caused by deficiency of lysosomal acid alpha glucosidase (GAA) in skeletal muscle cells of Pompe patients *in vitro*. To this end, we cloned the native *GAA* cDNA in the donor construct. iPSCs were generated from

Figure 2. *In Vitro* Expansion, Differentiation, and Maturation of Purified Myogenic Progenitor Cells

(A) Proliferation curves of myogenic progenitors derived from 15 iPSC lines derived from healthy controls or Pompe patients, cultured in proliferation medium. An exponential trend line was plotted and an R^2 was calculated from all data points, which showed similar proliferation rates for all cell lines.

(B) Recovery of control 1 myogenic progenitors from freezing. Data are means ± SD from three independent cultures.

(C) Average cell cycle duration of all cell lines shown in (A). Data are means ± SD of all cell lines shown in (A).

(D) After expansion for the number of days indicated on the X axis, skeletal muscle differentiation was induced for 4 days by switching to differentiation medium. The fusion index was quantified after staining for MHC and Hoechst. Individual values of random fields per cell line (n = 3–5 fields per cell line) are plotted as symbols. Mean values of all cell lines per expansion period are indicated as horizontal lines.

(E) At day 8 of differentiation, myotubes further matured as indicated by staining for fast MHC, MHC, titin, and α-actinin, a striated pattern, and spontaneous contractions (see Videos S1 and S2). Blue, nuclei as stained with Hoechst.

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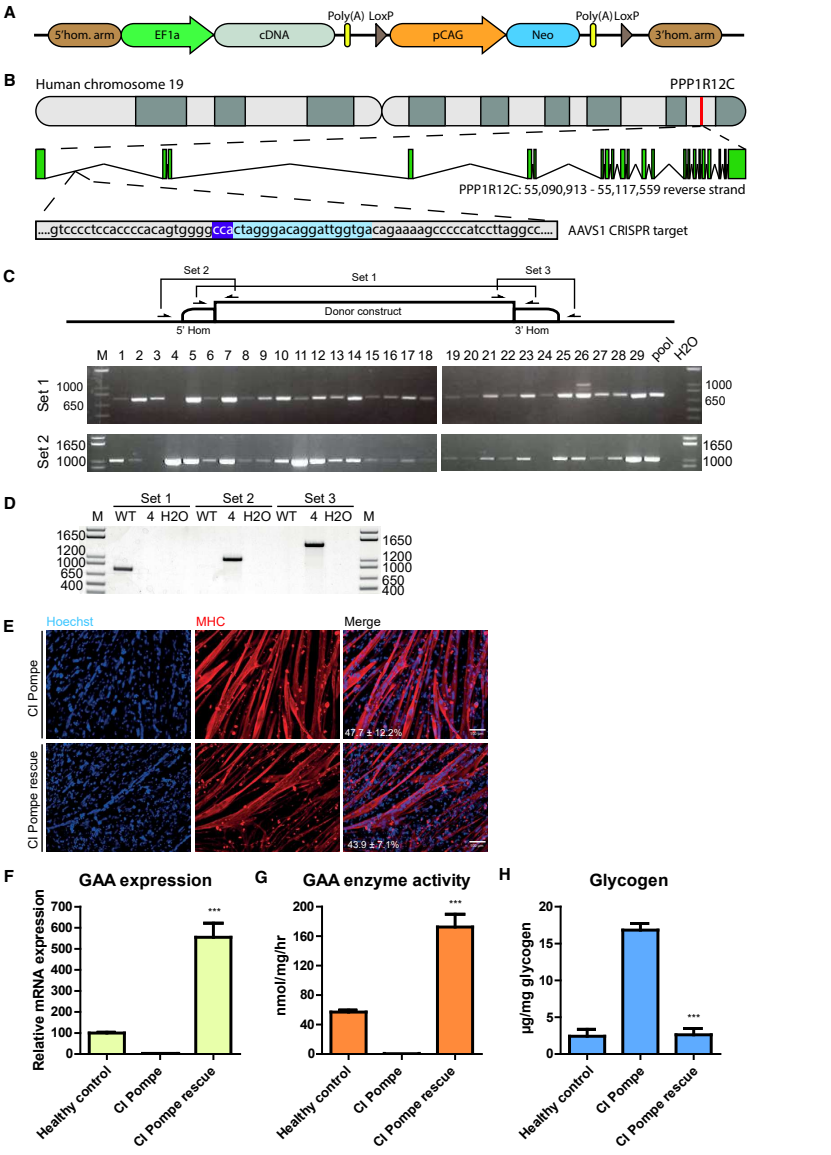

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a patient with classic infantile (CI) Pompe disease (the most severe phenotype, which is characterized by complete deficiency of GAA enzyme activity), and co-transfected the donor vector containing the *GAA* cDNA with vectors that expressed a guide RNA targeting the AAVS1 locus and a human codon-optimized Cas9 nuclease. After selection with G418, an average of 200 colonies were obtained per 2×10^6 cells, suggesting a targeting frequency of 1×10^{-4}%. Twenty-nine colonies were picked and genotyped using two PCR strategies (Figure 3C). With PCR primer set 1, the untargeted allele yields a product of 749 bp, while the targeted allele yields a product that is too large to be amplified under the conditions employed. With primer set 2, insertion of the *GAA* cDNA at the correct location is detected. The results with primer set 2 showed that 27/29 colonies had inserted the *GAA* cDNA at the desired location. With primer set 1, 28/29 colonies showed that the second allele had not been targeted. One colony (clone 4) contained two targeted alleles. iPSCs from clone 4 were expanded, and the correct integration site was further validated at the 3′ site using primer set 3 (Figure 3D). iPSCs from clone 4 were expanded, and myogenic progenitors were generated and compared with myogenic progenitors from the original iPSC line before gene editing. Myogenic progenitors from these lines were purified, expanded, and subjected to myotube differentiation. Similar differentiation capacities and fusion indexes were observed before and after gene editing (Figure 3E). RT-qPCR analysis showed the absence of *GAA* mRNA expression in the untargeted Pompe myotubes; this was caused by mRNA decay following a frameshift in both alleles (*GAA* genotype c.525del/c.525del). In the gene-edited myotubes, *GAA* mRNA expression had been restored 5.5-fold over levels in healthy control myotubes (Figure 3F). GAA enzyme activity measurements showed complete restoration of GAA activity in the gene-edited myotubes to levels that were ~3-fold higher than those of healthy control myotubes (Figure 3G). Myotubes from the CI Pompe patient showed accumulation of glycogen that was restored in the gene-edited myotubes to the levels of healthy control myotubes (Figure 3H). Altogether, these results demonstrate the feasibility of combining gene editing in iPSCs with the myogenic differentiation protocol to generate gene-corrected skeletal muscle cells.

Expression Profiling of iPSC-Derived Myogenic Progenitors

To characterize myogenic progenitors, we used RNA sequencing (RNA-seq) to perform genome-wide mRNA expression analysis. Profiles from purified, expanded (~15 days) iPSC-derived myogenic progenitors from healthy controls were compared with publicly available datasets (see Table S3) on cell types of different lineages, including adult SCs (FACS purified), myoblasts/myosatellite cells (prepared using preplating), neuronal cells, chondrocytes, cardiomyocytes, hepatocytes, embryonic stem cells, smooth-muscle cells, mesenchymal stem cells, and fibroblasts (Figure 4A). The "new Tuxedo" pipeline (Pertea et al., 2016) was used. Spearman correlation analysis showed that profiles of two independent biological replicates of myogenic progenitors from independent individuals clustered together, indicating that these cells contained similar and defined gene expression profiles (Figure 4, myogenic progenitors from the present study are indicated in green). The profiles of myogenic progenitors clustered away from all other cell types, while the profiles of the adult quiescent and activated muscle stem cells showed an early split from all other profiles. A total of 1,852 out of 13,193 genes were differentially expressed between activated muscle stem cells and myogenic progenitors (false discovery rate < 0.01; Table S5). The dissimilarity between quiescent and

Figure 3. Gene Editing in iPSCs Restores the Pompe Disease Phenotype in Skeletal Muscle Cells *In Vitro*

(A) Generic construct for insertion of a cDNA in a safe harbor following CRISPR/Cas9-mediated targeting.

(B) The construct shown in (A) was tailored to express *GAA* in the AAVS1 locus. After transfection into iPSCs from a classic infantile (CI) Pompe patient, G418 selection was used, and single colonies were picked.

(C) Genotyping was performed using PCR. Primer sets 2 and 3 amplified a product that is only present in correctly targeted clones, while primer set 1 spanned the insertion site to give a product only in the absence of targeting. With primer set 1, 28/29 clones were positive, indicating that most clones also contained an untargeted allele; with primer set 2, 27/29 clones were positive, indicating that most clones showed efficient targeting of at least one allele.

(D and E) (D) One clone (#4) showed targeting of both alleles, which was validated using primer set 3, and was differentiated into myogenic progenitors for further analysis. Myogenic progenitors were generated from healthy controls, a CI Pompe patient (CI Pompe), and the isogenic, gene-corrected, CI Pompe patient (CI Pompe rescue). Myogenic progenitors were purified, expanded, differentiated for 6 days into myotubes, and the fusion index was determined (E).

(F–H) Myogenic progenitors were analyzed for *GAA* mRNA expression at day 4 (F); GAA enzyme activity at day 4 (G); and glycogen accumulation at day 6 (H). *GAA* mRNA expression was measured by RT-qPCR using primers spanning exon 1–2. GAA enzyme activity was measured using the 4-methylumbelliferone assay. Glycogen accumulation was measured biochemically. To deplete cytoplasmic glycogen, cells were cultured in glucose-free medium for the last 24 hr, as described in Bergsma et al. (2015). For (F, G, and H), data are means ± SD of two independent (healthy control) or three independent (CI Pompe disease and rescue) cultures. Two-tailed Student's t test: ***$p < 0.001$.

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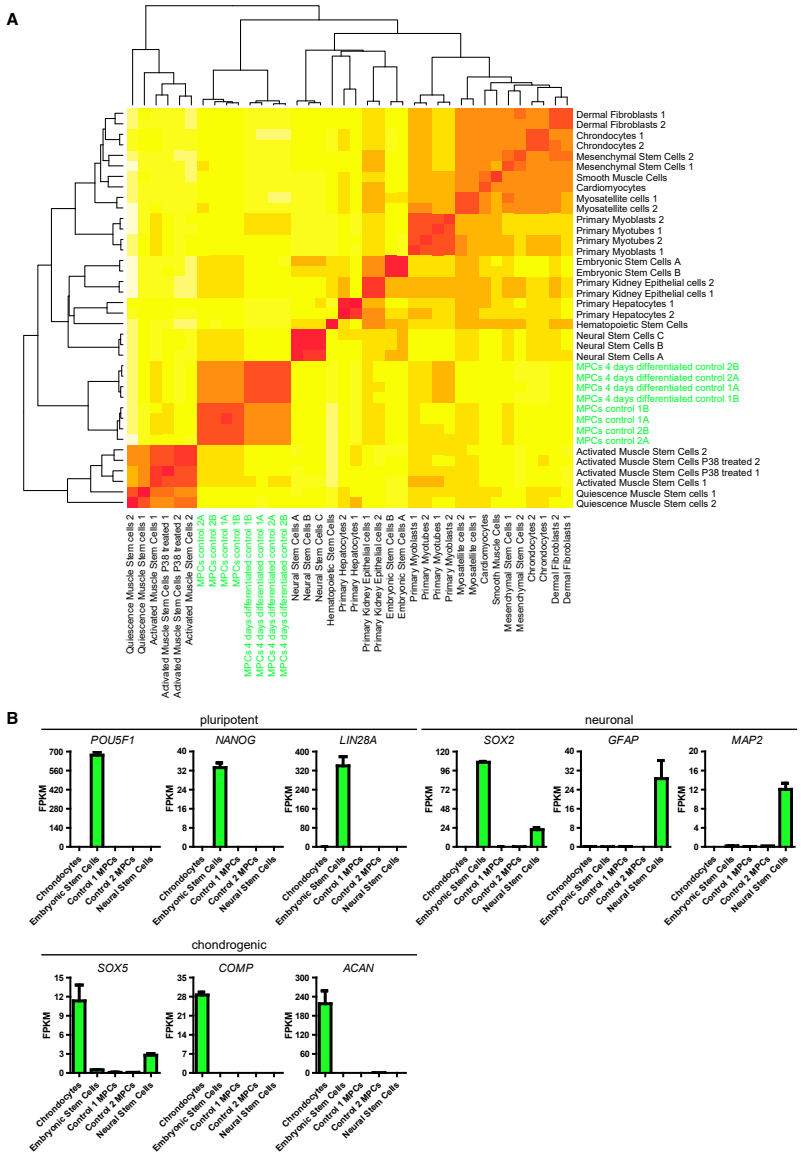

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activated muscle stem cells from myosatellite cells and primary myoblasts can be explained by the fact that the former cells were FACS purified, while the latter cells were obtained using preplating and probably contained contaminating cell types. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis of genes that were differentially expressed in myogenic progenitors relative to activated muscle stem cells showed enrichment of the AMPK, MAPK, and ErbB signaling pathways in myogenic progenitors (Figure S2C). These pathways have been involved in cell cycle regulation, muscle regeneration, and/or satellite cell function (Charville et al., 2015; Golding et al., 2007; Theret et al., 2017). Overall, this suggests that the myogenic progenitors were dissimilar from the other cell types tested and contained a defined mRNA expression profile.

To assess the purity of the myogenic progenitors, we used the datasets shown in Figure 4A to examine the expression of markers for pluripotent cells (*POU5F1*, *NANOG*, and *LIN28A*), neuronal cells (*SOX2*, *GFAP*, and *MAP2*), and chondrogenic cells (*SOX5*, *COMP*, and *ACAN*). None of these markers were expressed in the purified iPSC-derived myogenic progenitor cultures, suggesting that contaminating cells from the lineages tested were absent (Figure 4B).

In earlier work we showed that, upon expansion, purified iPSC-derived myogenic progenitors express several myogenic markers, including the MyoD protein (van der Wal et al., 2017b). To examine PAX7 protein expression during *in vitro* expansion and differentiation, we used a PAX7 antibody to perform immunofluorescent analysis. Under proliferating conditions, expanded myogenic progenitors (~25 days) from two independent iPSCs expressed PAX7 in a subset of cells (Figure 5A). Although myogenic progenitor cultures contained a stable ~3% of $PAX7^+$ cells during the majority of the expansion period, the percentage of $Pax7^+$ cells started to decline at day 39 (control 1) or day 28 (control 2) (Figure 5B). After differentiation to myotubes, $PAX7^+$ cells remained present in the culture (Figure 5C and data not shown). These results indicate that, during expansion, a subset of iPSC-derived myogenic progenitors continue to express markers of SCs during both proliferation and differentiation.

In Vivo Myogenic Potential of Myogenic Progenitors

To test the capacity of purified and expanded myogenic progenitors to engraft and contribute to muscle regeneration *in vivo*, we performed cell transplantations in tibialis anterior (TA) muscles of NSG immunodeficient recipient mice that had been pre-injured with $BaCl_2$. Analysis of engraftment was performed 4 weeks after transplantation. Using human-specific epitopes (Lamin A/C, Spectrin, and Dystrophin; for controls, see Figure S3A), we observed that myogenic progenitors that had been expanded for 3 days were able to engraft and participate in the formation of new myofibers (Figure 6A). In addition, myogenic progenitors were engrafted after longer periods of expansion (6 and 11 days), and at different cell concentrations (2.5×10^5 to 1×10^6, healthy control 1 line) (n = 6 mice) (data not shown). Quantification of the number of $Spectrin^+$ fibers showed that cell engraftment efficiency was 35–58 fibers/section, with 87–127 Lamin A/C^+ nuclei/section (Figure 6B, using two independent cell lines: control 1 and control 5). Lamin A/C^+ nuclei were found within myofibers and in the interstitium. A subset of Lamin A/C^+ nuclei was found at a satellite cell position (Figure S3B top); however, very few of those were $Pax7^+$ (Figure S3B bottom). The location of Lamin A/C^+ nuclei was as follows: ~45% was found within human $Spectrin^+$ myofibers, suggesting that these contributed to myofiber formation (Figure 6C); 25%–36% was found in the interstitium (Figure S3C); the remaining 23%–40% was found within $Spectrin^-$ myofibers, which may indicate that in those (multinucleated) fibers mouse nuclei were dominant. These results demonstrate the engraftment potential and regenerative capacities of expanded myogenic progenitors and their participation in muscle regeneration *in vivo*.

DISCUSSION

In this study, we have characterized FACS-purified myogenic progenitors for their applicability *in vitro* and *in vivo*, and provide a detailed protocol to generate these cells. The principle of the procedure and its possible applications are shown in Figure 7. We showed that it is possible to reproducibly generate myogenic progenitors from 15

Figure 4. Molecular Profiling and Purity of Myogenic Progenitors

(A) Purified myogenic progenitors have a myogenic gene expression signature. Heatmap showing a comparison of genome-wide mRNA expression (as measured by RNA-seq) from myogenic progenitors and publicly available datasets. Purified myogenic progenitors from two healthy control iPSCs were included: cells were either expanded for ~15 days in proliferation medium or differentiated for 4 days. Published datasets are listed in Table S3. Datasets were analyzed using the "new Tuxedo" pipeline as described in Pertea et al. (2016). Spearman correlations are shown. Datasets generated in this study are indicated in green.

(B) Purified myogenic progenitors do not express pluripotency markers (*POU5F1*, *LIN28A*, and *NANOG*), neuronal markers (*SOX2*, *GFAP*, and *MAP2*), or chondrogenic markers (*SOX5*, *COMP*, and *ACAN*). Data were extracted from (A). Data are means ± SD of two independent cultures per cell line.

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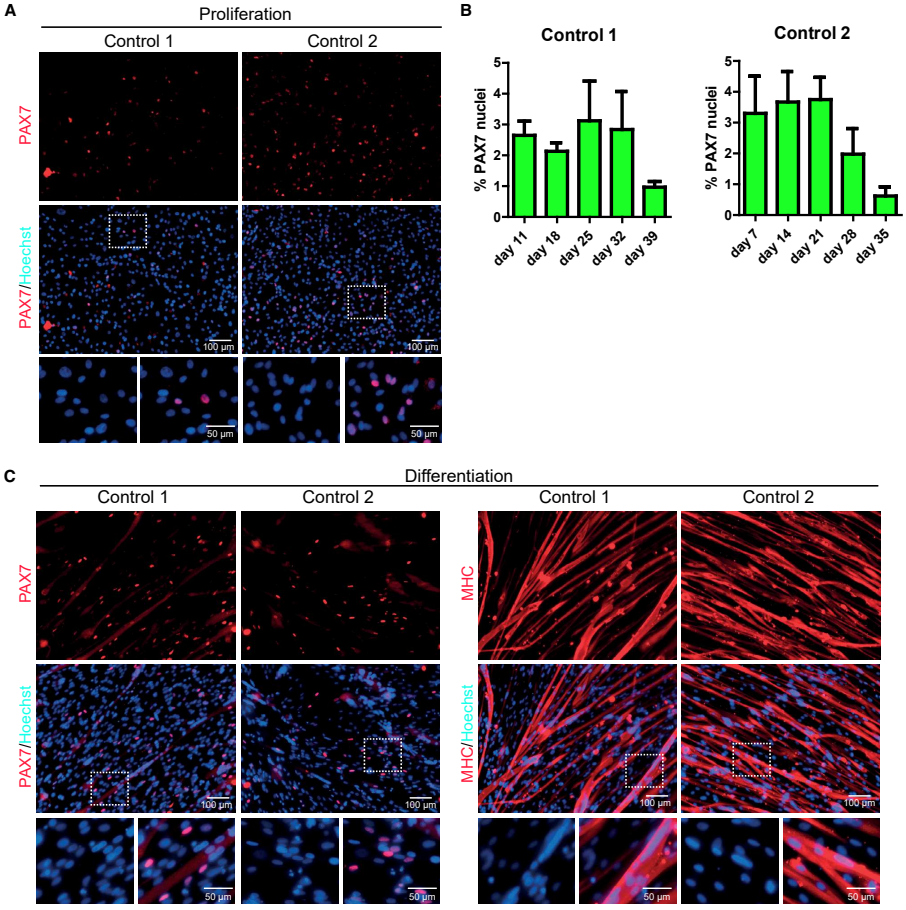

Figure 5. PAX7 Expression during *In Vitro* Proliferation and Differentiation of Purified Myogenic Progenitors

(A) Purified myogenic progenitors from two healthy control iPSCs were expanded for ~25 days in proliferation medium and stained with a PAX7 antibody and Hoechst to stain nuclei.

(B) Quantification of PAX7$^+$ cells during expansion of myogenic progenitors from the two healthy control iPSCs shown in (A). Data are means ± SD of n = 5 fields per point.

(C) Myogenic progenitors were differentiated for 6 days to myotubes. Immunofluorescent analysis was performed using a PAX7 antibody (in red) or an MHC antibody (in red) to monitor myotube formation, as indicated. Nuclei were stained with Hoechst (blue).

iPSC lines that were derived from different donors. As we have shown previously, 4×10^4 sorted myogenic progenitors could be expanded to as much as 1×10^{12} cells within 31 days without losing differentiation capacity (van der Wal et al., 2017b). Our current data show that the period during which myogenic progenitors can be expanded can

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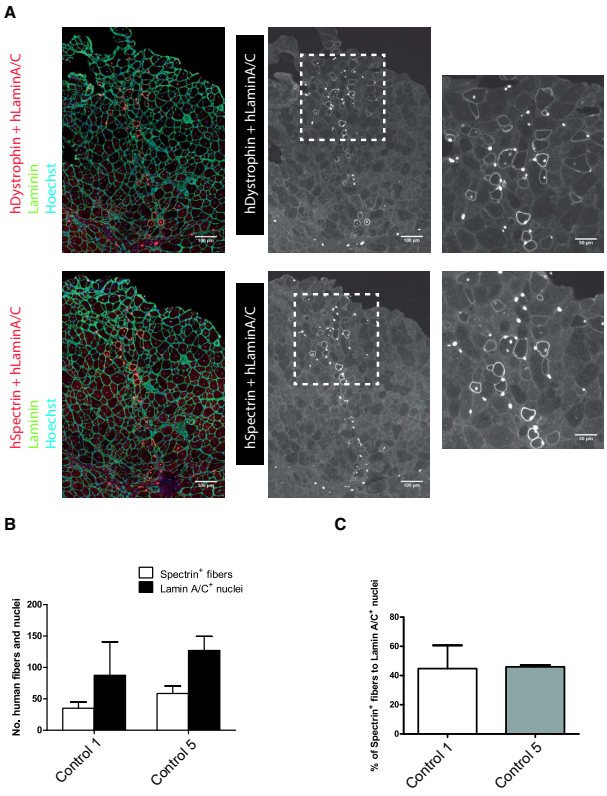

Figure 6. *In Vivo* Myogenic Potential of Purified Myogenic Progenitors Following Engraftment in Immunodeficient Mice

(A) Twenty-four hours before transplantation, the TA of NSG mice was injured using $BaCl_2$. Myogenic progenitors were administered using intramuscular injection of 5×10^5 cells. Four weeks after transplantation, engraftment was determined by immunohistochemistry of human-specific Lamin A/C and Dystrophin or Spectrin (white or red) and multi-species Laminin (green) on consecutive cross sections.

(B and C) (B) Quantification of Spectrin$^+$ muscle fibers and Lamin A/C$^+$ nuclei and (C) the percentage of Spectrin$^+$ fibers relative to the total number of Lamin A/C$^+$ nuclei per section of each biological replicate. Data in (B) and (C) are means ± SD (n = 2 TAs transplanted per line used. Each replicate was transplanted in different mice). All sections were counterstained with Hoechst (blue). Scale bars represent 100 μm, and 50 μm on insets.

be extended to up to 43 days. After ~50 days of expansion, changes in morphology and proliferation rate suggested the initiation of a senescent phenotype. It is therefore likely that, during the expansion, myogenic progenitors slowly progress to a myoblast-like phenotype, a cell type that is known to undergo replicative senescence during passaging (Bigot et al., 2008). After 43 days of culture, myogenic progenitors had expanded as much as 5×10^{11}-fold (the maximum value obtained), allowing the generation of at least 2×10^{16} cells, which should be sufficient for subsequent analyses, including high-throughput screenings and engraftment studies.

We generated a generic donor construct that can be used for precise and highly efficient gene correction. The selection of positive clones is facilitated by its inclusion of a selection marker. The option of removing the selection marker using transient CRE recombinase expression may be useful in future *in vitro* and *in vivo* applications. A prerequisite for the strategy of inserting a wild-type copy of a cDNA of interest is that overexpression of the transgene product should not be harmful; if it is, the choice of promoter that drives the transgene should be optimized. Overexpression of a transgene is expected to benefit the development of cell-based therapeutic strategies. In the case of skeletal muscle, which consists of multinucleated cells, it can be envisioned that overexpression in a subset of engrafted myonuclei that become part of the syncytium of the affected myofibers would cross-correct part of the myofiber. If the transgene product is secreted, as GAA is secreted in Pompe disease, overexpression potentially

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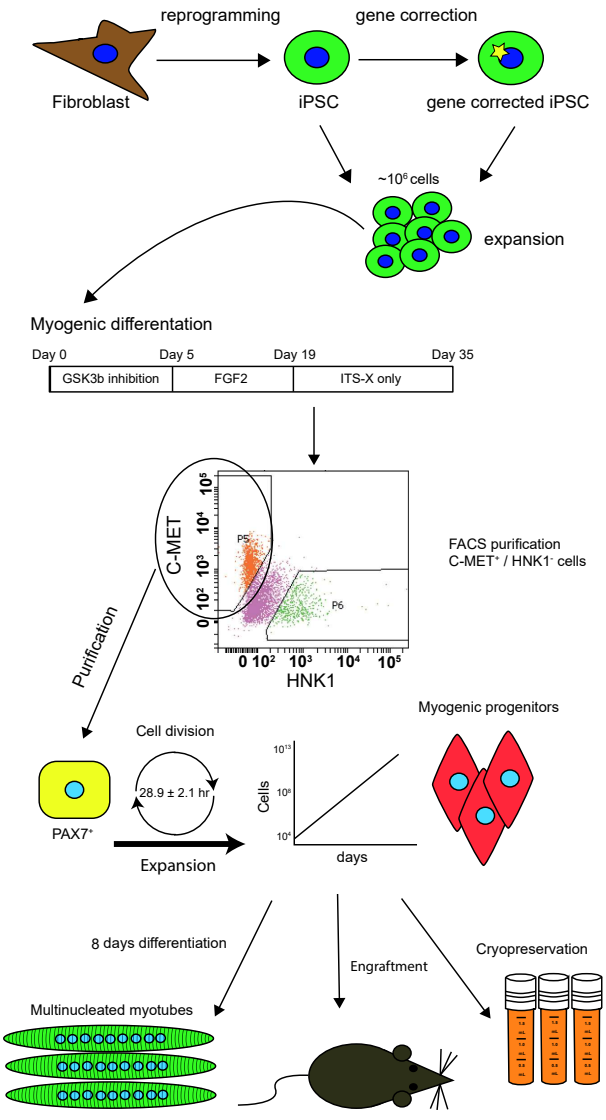

Figure 7. Cartoon Highlighting the Applications of Myogenic Progenitors Described Here

Human iPSCs derived from healthy controls or patients are used as starting cells. Gene correction is applied to iPSCs using CRISPR/Cas9-mediated insertion of a cDNA into a safe harbor. Original or gene-corrected iPSCs are differentiated into the myogenic lineage using a 35-day transgene-free protocol. Myogenic progenitors are purified using a 1-step FACS procedure, and are then expanded (up to 5×10^{11}-fold) and cryopreserved. During expansion, purified myogenic progenitors are differentiated *in vitro* into myotubes with high fusion index, and show striation and spontaneous contraction upon *in vitro* maturation. Upon engraftment in immunodeficient mice, purified and expanded myogenic progenitors form human mononuclear cells and contribute to myofiber formation *in vivo*.

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results in cross-correction of neighboring myofibers (Zaretsky et al., 1997).

Both iPSC-derived myogenic progenitors and SCs express PAX7 during *in vitro* proliferation and differentiation, and contribute to myofiber formation after intramuscular engraftment in immunodeficient mice *in vivo*. We showed that, upon optimization of the differentiation process using defined medium conditions, the cells not only expressed fast MHC, α-actinin, and titin but also formed functional sarcomeres, thereby allowing spontaneous contractions. These data revealed enhanced maturation compared with that found in our previous report (van der Wal et al., 2017b) and showed that it is possible to generate mature myofibers from purified iPSC-derived cultures. The formation of myogenic progenitors from Pompe patient-derived iPSCs was not hampered by the underlying disorder, and we expect that this approach can be used to model other disorders that affect muscle cells. It remains to be determined whether, as Chal et al. (2015) report, purified myogenic progenitors have the capacity to differentiate into millimeter-long skeletal muscle cells with $PAX7^+$ cells embedded between the sarcolemma and the basal lamina.

It is essential for the development of stem cell-based therapies that the transplantable cell preparations are highly pure and well characterized before transplantation in human patients can be considered. The development of techniques for the expansion and manipulation of pure myogenic progenitor populations *ex vivo* is therefore critical to the further development of this field. In this paper we have provided evidence for the successful engraftment of myogenic progenitors in pre-injured muscles of mice over a period of 4 weeks post-transplantation. The efficiencies of engraftment of mononuclear cells and their contribution to myofibers were comparable with those recently obtained using inducible PAX7 overexpression (Magli et al., 2017). Transplanted myogenic progenitors demonstrated their ability to regenerate injured muscle, as was shown by the detection of centrally located lamin A/C^+ human nuclei, a characteristic perceived only in fusion-competent myoblasts.

Future studies should identify the stem cell properties of transplanted human myogenic progenitors that allow transplanted donor cells to make a long-term contribution to muscle regeneration. This would provide researchers with novel tools that would help them make progress in the development of muscle stem cell therapies for treating muscle-wasting diseases.

EXPERIMENTAL PROCEDURES

Ethics Approval and Consent to Participate

The Institutional Review Board approved the study protocol, and all patients provided written informed consent. All animal experiments were approved by the animal experiments committee DEC-Consult.

Culture of Myogenic Progenitors

Myogenic progenitors were expanded in myogenitor progenitor proliferation medium consisting of DMEM high glucose (Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (Hyclone, Thermo Scientific, Waltham, MA), 1% penicillin-streptomycin-glutamine (P/S/G) (Gibco, Waltham, MA), and 100 ng/mL FGF2 (Prepotech, Rocky Hill, NJ) on extracellular matrix-coated dishes (1:200 diluted, Sigma-Aldrich, E6909). For splitting, myogenic progenitors were detached with TrypLe reagent (Gibco, Waltham, MA) diluted 2× with PBS (Gibco, Waltham, MA). For cryopreservation, myogenic progenitors were detached as described above, and after centrifugation the cell pellet was resuspended in myogenic progenitor proliferation medium supplemented with 10% DMSO. Standard cell culture techniques were used for the freeze and thaw procedure.

RNA Isolation and RNA-Seq

Myogenic progenitors were expanded for ~15 days and harvested either in proliferation conditions or after 4 days of differentiation as described previously (van der Wal et al., 2017b). RNA was extracted using the RNeasy minikit with DNase treatment (QIAGEN, Germantown, MD). Sequencing libraries were prepared using TruSeq Stranded mRNA Library Prep Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Libraries were sequenced on a HiSeq2500 sequencer (Illumina, San Diego, CA) in rapid-run mode according to the manufacturer's instructions. Reads 50 bp in length were generated. The RNA-seq datasets listed in Table S3 were downloaded and aligned with the datasets generated in this study using the new Tuxedo pipeline as described by Pertea et al. (2016). Shortly, RNA-seq data were aligned using Hisat2 (version 2.1.0) to hg38 from University of California, Santa Cruz. The alignments were converted to BAM format using Samtools (version 1.3.1). Then, StringTie was used to quantify transcript expression levels according to the reference transcripts. For KEGG analysis, gene expression was quantified using StringTie with the -e option.

Maturation of Myogenic Progenitors into Skeletal Muscle Cells

When myogenic progenitors reached 90% confluence, cells were switched to myogenic progenitor differentiation medium containing DMEM high glucose supplemented with 1% P/S/G, 1× ITS-X, and 1% knockout serum replacement (all Gibco). Medium was not refreshed during differentiation and cells were harvested at 6 days, 8 days, or 12 days.

Construction of Donor Vector

To generate the generic donor vector for the overexpression of the gene of interest via CRISPR/Cas9-mediated knockin, we used the pCAGEN and pEF-GFP vectors (available on addgene: #11160 and #11154) as starting points. The neomycin selection cassette was introduced via PCR amplification into the pCAGEN vector, destroying the EcoRI and NotI sites. KpnI and ClaI sites were then added to the SalI site, and loxP and SfuI sites were added to the HindIII site. In the pEF-GFP vector, a KpnI site was added to the SalI site and loxP and ClaI sites were added to the HindIII site. Vectors were

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combined using the KpnI and ClaI sites. The *GAA* cDNA was introduced via PCR amplification with EcoRI and NotI fragments. The 5′ homology arm of 700 bp was added via PCR amplification with KpnI fragments, and the 3′ homology arm of 972 bp was added via PCR amplification with HindIII fragments. All constructs were validated by sequencing. Cloning details are available on request.

Glycogen Assay

Myogenic progenitors were differentiated for 6 days in myogenic progenitor differentiation medium. On day 5 of differentiation, skeletal muscle cells were starved with differentiation medium without glucose (DMEM no glucose, Gibco). On day 6, skeletal muscle cells were detached with a scraper and the pellet was lysed with ice-cold protein lysis buffer (see Supplemental Experimental Procedures). Glycogen was measured as described in Bergsma et al. (2015).

Gene Editing of iPSCs

To select optimal target sites for the AAVS1 locus, single guide RNA (sgRNA) sequences were designed using the CRISPRscan program (Moreno-Mateos et al., 2015). The sgRNA CCACTAGGGACAGGATTGGTGA was expressed from a TOPO vector containing the U6 promoter (addgene: 41824). Confluent iPSCs on feeders were pretreated 4 hr before nucleofection with 10 μM Rock inhibitor (Y-27632 dihydrochloride, Ascent Scientific, Asc-129). Single cells were generated from iPSC colonies by incubating with Accutase (Thermo Scientific, Waltham, MA), and 2×10^6 cells were nucleofected with 4 μg of pCAG-hCAS9-GFP (addgene: 44719), 3 μg of TOPO-sgRNA, and 2 μg of donor vector using Amaxa Human Stem Cell Nucleofector Kit2 (VPH-5022, Lonza, Walkersville, MD) with program B-016. After nucleofection, cells were recovered in iPSC-conditioned medium (iPSC medium incubated for 24 hr on feeder cells) supplemented with 20 ng/mL FGF2 (Prepotech, Rocky Hill, NJ) and 10 μM rock inhibitor. iPSCs were selected after 48 hr of nucleofection with 100 μg/mL G-418 (Invivogen, San Diego, CA). Approximately 14 days after selection of the iPSCs, single colonies were picked and genotyped using primers from Table S4.

Transplantation into NSG Mice

NSG (Jackson Laboratories) mice aged 2–6 months were used for transplantation studies. Mice (independently of gender) were anesthetized with isoflurane in oxygen from a vaporizer. Regeneration of skeletal muscle was induced by chemical injury. The endogenous skeletal muscle fibers of the mice were injured by injection with 50 μL of 1.2% barium chloride ($BaCl_2$) into the TA muscle. Twenty-four hours later, 20 μL of 5×10^5 dissociated cells were injected into the TA muscle in duplicates (one female and one male). Transplanted cells in this study were expanded for 3 days. PBS-injected TAs were used as negative control for cell transplantations. Mice were sacrificed 4 weeks after cell transplantation, and their TA muscles harvested. TA muscles were frozen in isopentane cooled in liquid nitrogen and stored at −80°C until analysis; 10 μm cryosections were obtained at intervals throughout the entire muscle and were either stored at −80°C for further immunostaining or were used immediately for PAX7 staining.

Immunofluorescent Stainings

Muscle cryosections were fixed in ice-cold acetone for 5 min, followed by a permeabilization step with 0.3% Triton X-100 in PBS for 20 min. Samples were incubated with a blocking solution of 20% goat serum (DAKO, Santa Clara, CA) and 2% BSA (Sigma-Aldrich, Irvine, UK) in 0.1% Tween in PBS for 1 hr. Sections were incubated with primary antibodies mouse anti-human Lamin A/C (1:100, VP-L550, Vector Laboratories, Burlingame, CA) plus mouse anti-human Spectrin (1:100, SPEC1-CE, Leica, Wetzlar, Germany) or mouse anti-human Dystrophin (1:150, MABT827, Millipore) co-stained with rabbit anti-Laminin (1:100, L9393, Sigma-Aldrich, Irvine, UK) overnight at 4°C. Tissue sections were stained with secondary antibodies goat anti-rabbit (Alexa Fluor 488, 1:500, A-21141, Life Technologies, Carlsbad, CA) and horse anti-mouse biotin (1:250, BA-2000, Vector Laboratories, Burlingame, CA) for 1 hr at room temperature, followed by incubation with Streptavidin 594 (1:500, S-32356, Invitrogen, Carlsbad, CA) for 30 min. Freshly cut tissue was used for PAX7 stainings. Sections were fixed in 4% paraformaldehyde for 5 min and blocked with 20% goat serum and 2% BSA in 0.5% Triton X-100 in PBS for 1 hr, then incubated with mouse anti-PAX7 (1/20, DSHB), Lamin A/C, and Laminin in blocking solution for 2 hr at room temperature. Goat anti-mouse IgG1 Cy3 (1:500, 115-165-205, Jackson ImmunoResearch), goat anti-mouse IgG2b Alexa Fluor 488 (1:500, A-21141, Thermo Fisher), and goat anti-rabbit Alexa Fluor 647 (1:500, A21245, Invitrogen, Carlsbad, CA) were used in 0.1% PBST for 1 hr. All sections were incubated with Hoechst nuclear staining (1:15,000 Invitrogen, Carlsbad, CA) for 10 min and mounted with Mowiol medium (Sigma-Aldrich, Irvine, UK). Images were obtained using confocal microscopy (Zeiss LSM 700).

Statistical Analysis

Data represent mean ± SD, and p values refer to two-sided t tests. Multiple groups were tested with one-way ANOVA followed by individual two-sided t tests. A p value of <0.05 was considered to be significant. Data showed normal variance and no samples were excluded from the analysis. Images for quantification were randomly selected.

ACCESSION NUMBERS

RNA-seq fastq files and data are accessible at GEO under GEO: GSE111163.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, five tables, and two videos and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.04.002.

AUTHOR CONTRIBUTIONS

Myogenic protocol, E.v.d.W., S.i.G., T.J.M.v.G., and W.W.M.P.P.; Engraftment, P.H.-H., T.J.M.v.G., G.J.S., and W.W.M.P.P.; Expression analysis, R.W., T.H.C., W.F.J.v.I., E.v.d.W., and W.W.M.P.P.; Gene edit and pathology, M.B., E.v.d.W., S.i.G., and W.W.M.P.P.; Funding, W.W.M.P.P., A.T.v.d.P., and G.J.S.; Data interpretation,

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all authors; Writing, E.v.d.W., P.H.-H., and W.W.M.P.P.; Supervision, G.J.S., T.H.C., and W.W.M.P.P.

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Stem Cell Reports, Volume *10*

Supplemental Information

Large-Scale Expansion of Human iPSC-Derived Skeletal Muscle Cells for Disease Modeling and Cell-Based Therapeutic Strategies

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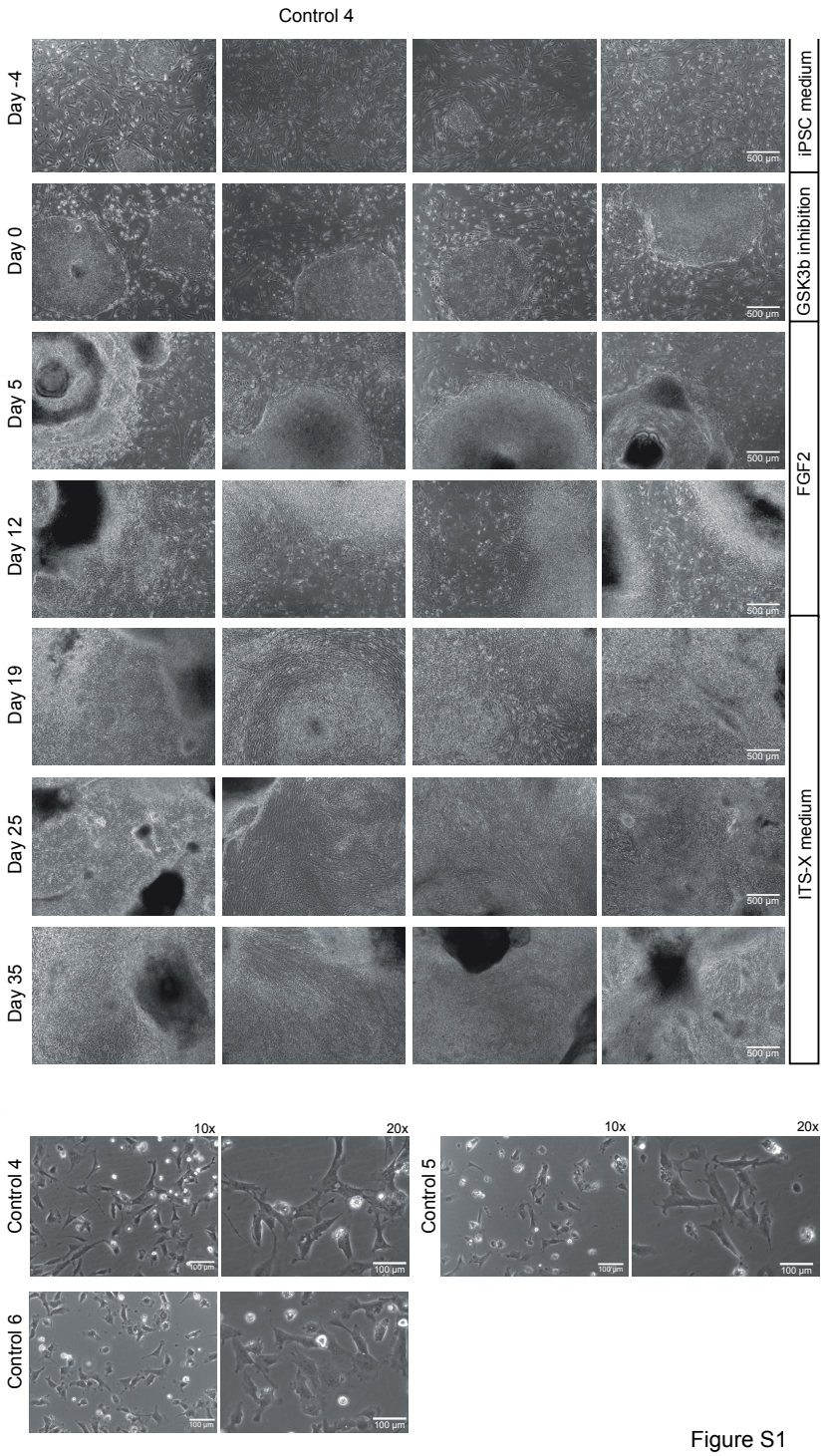
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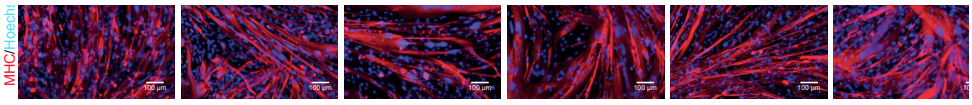
100 µm

Figure S1



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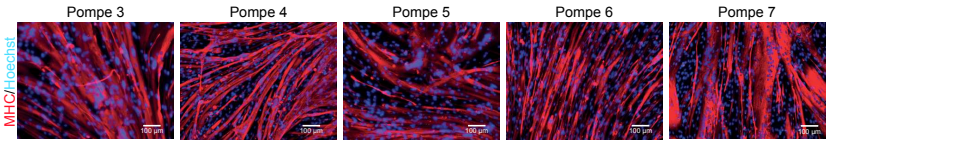
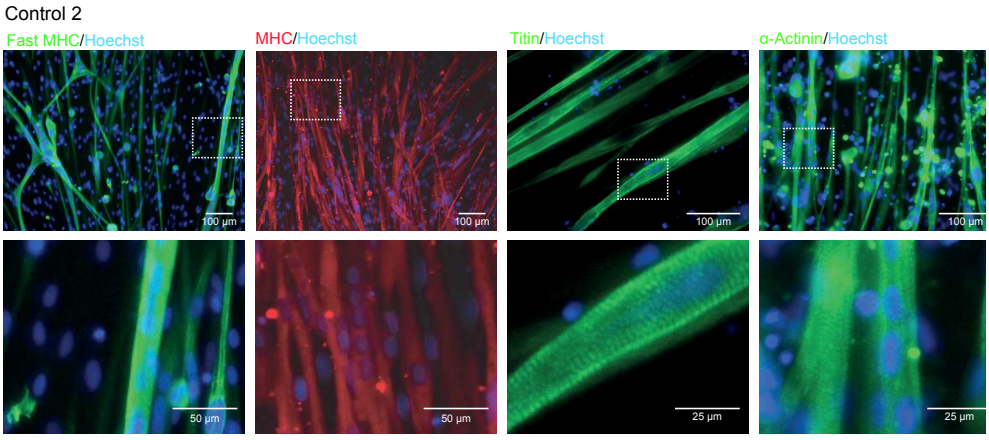
18d	69,4%	4,8%	37,3%	1,4%	37,9%	12,1%	40,1%	11,1%	51,8%	14,4%	62,7%	13,4%
25d	54,3%	16,2%	46,0%	8,6%	57,9%	7,8%	81,9%	6,3%	48,4%	20,5%	50,8%	2,3%
35d	31,4%	6,8%	49,5%	7,0%	64,1%	3,6%	79,6%	4,0%	35,3%	1,7%	41,7%	4,3%



	Pompe 3		Pompe 4		Pompe 5		Pompe 6		Pompe 7	
	average	sd	average	sd	average	sd	average	sd	average	sd
-14d	53,6%	27,0%	54,1%	8,2%	44,8%	10,4%	43,3%	7,8%	33,0%	7,7%
-18d	45,8%	9,7%	42,3%	4,5%	44,3%	7,3%	49,4%	4,1%	52,5%	1,3%
-25d	77,2%	13,7%	49,8%	7,0%	59,0%	19,2%	36,8%	8,3%	53,4%	6,6%
-35d	65,1%	12,9%	37,9%	8,1%	43,6%	2,1%	35,5%	8,7%	38,7%	6,7%



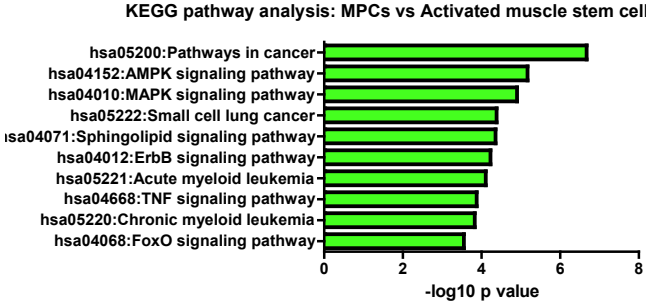


Figure S2

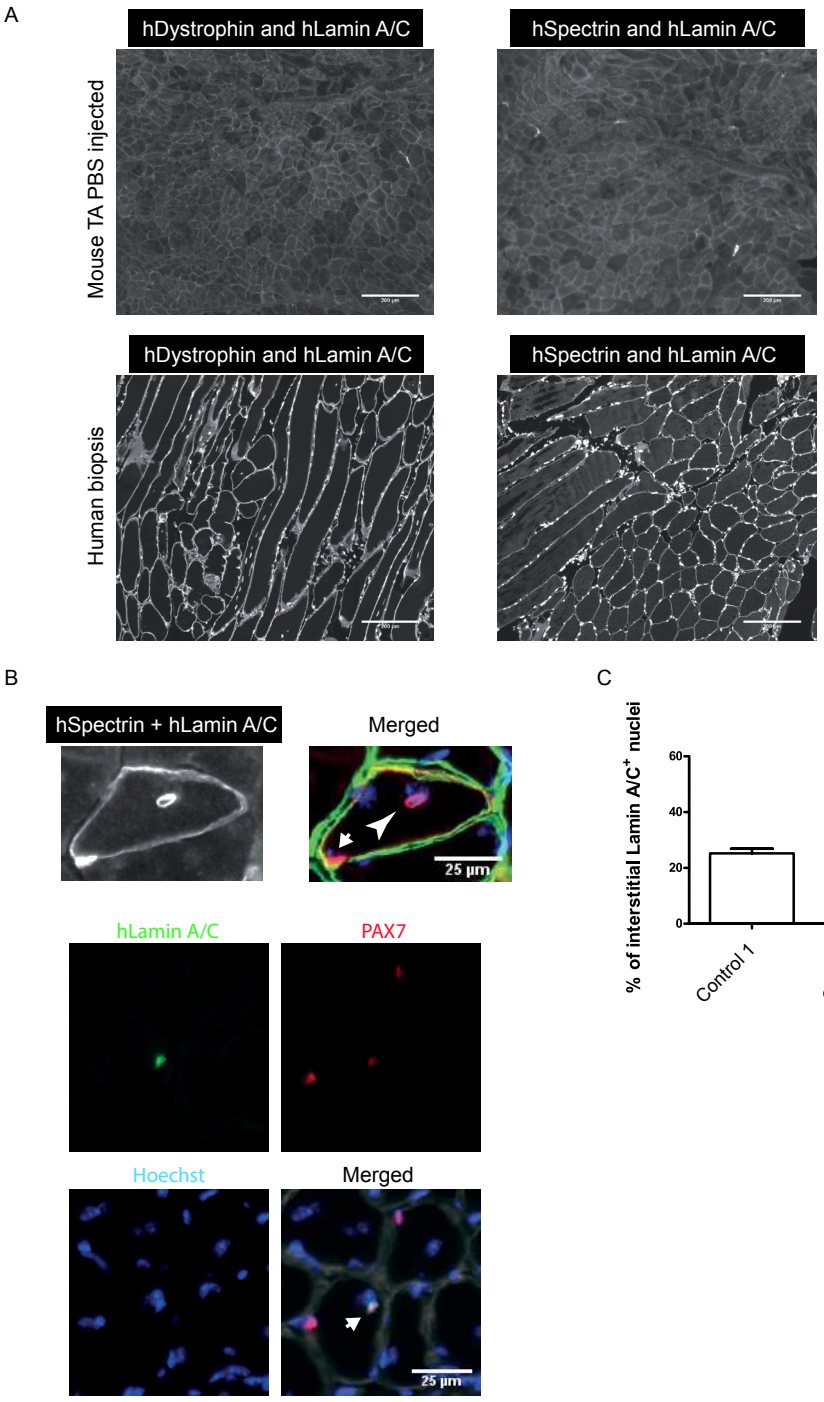

A
hDystrophin and hLamin A/C
hSpectrin and hLamin A/C
Mouse TA PBS injected
hDystrophin and hLamin A/C
hSpectrin and hLamin A/C
Human biopsis
B
hSpectrin + hLamin A/C
Merged
25 µm
hLamin A/C
PAX7
Hoechst
Merged
25 µm
C
% of interstitial Lamin A/C+ nuclei
60
40
20
0
Control 1
Control 5

Figure S3

SUPPLEMENTARY FIGURE LEGENDS

Figure S1 (related to Figure 1): Cell morphologies during differentiation of iPSCs into the myogenic lineage and after purification of myogenic progenitors. (A) Healthy-control iPSCs 3, 4, 5 and 6 were differentiated using a 35-day protocol consisting of GSK3β inhibition, FGF2 treatment, and a minimal medium (see Figure 7). Light microscope (4x magnification) images were taken before and during differentiation at the days indicated. Representative images are shown. (B) One day after FACS purification (described in Figure 7), light microscope images were taken from myogenic progenitors generated from healthy controls 4, 5 and 6 at a magnification of 10x and 20x. Representative images are shown.

Figure S2 (related to Figures 2 and 4): Differentiation, maturation of purified myogenic progenitors, and KEGG pathway analysis. (A) Myosin heavy chain (MHC) staining on 4 days differentiated myogenic progenitors from healthy controls 3- 8 and Pompe 3 – 7. For images of healthy controls 1 and 2, and Pompe 1 and 2, see van der Wal et al., 2017. Nuclei were stained with Hoechst. Images are representative for each differentiation. Fusion index during expansion was quantified and data are mean ± SD of 3 fields per point. (B) Staining of matured fibers from myogenic progenitors of healthy control 2. After 6-8 days of differentiation, cells were stained with Fast MHC, MHC, Titin and α-Actinin antibodies. Nuclei were stained with Hoechst and are shown in blue. (C) KEGG pathway analysis using DAVID of mapped genes comparing myogenic progenitors in proliferation phase (MPCs, this study) versus activated muscle stem cells (Charville et al., 2015). The 10 most significant pathways are shown.

Figure S3 (related to Figure 6): Positive and negative controls for analysis of *in vivo* engraftment and for cell contribution to muscle regeneration *in vivo*. (A) The upper panels show sections from the *tibialis anterior* from immunodeficient mice 4 weeks after injection with PBS only. The lower panel shows sections from a human biopsy of the quadriceps femoris. Sections were analyzed by immunohistochemistry using human specific Lamin A/C, Spectrin and Dystrophin antibodies (white). (B) Upper panel represent two different locations of Lamin A/C$^+$ nuclei within the same Spectrin$^+$ fiber. The human nuclei on a satellite cell position are indicated with an arrow and the myonuclei with an arrowhead. Lower panel shows a PAX7$^+$ (red), Lamin A/C$^+$ (green) nucleus in a Laminin$^+$ (grey) muscle fiber. (C) Percentage of Lamin A/C$^+$ nuclei present at the muscle interstitium per section of each biological replicate. Sections that showed engraftment were used for quantification. Data are mean ± SD (n= 2 TAs transplanted per line used. Each replicate was transplanted in different mice). All sections were counterstained with Hoechst (blue).

SUPPLEMENTARY TABLES

Table S1. Comparison of transgene-free skeletal-muscle differentiation protocols using GSK3β inhibition

	Purification protocol	**Fold expansion**	**Cryopreservation**	**Duration**	**Fusion index**	**Engraftment**
(Borchin et al., 2013)	FACS	N.R.	N.R.	35 days	N.R.	N.R.
(Xu et al., 2013)	No purification, differentiation analysed in original plate	N.R.	N.R.	36 days	N.R.	Yes, unpurified culture
(Shelton et al., 2014)	No purification, differentiation analysed in original plate	N.R.	N.R.	50 days	N.R.	N.R.
(Chal et al., 2015)	No purification, differentiation analysed in original plate	N.R.	N.R.	50 days	N.R.	N.R.
(Shelton et al., 2016)	No purification, differentiation analysed in original plate	3x	N.R.	50 days	N.R.	N.R.

(Choi et al., 2016)	FACS	10^5x	Yes	30 days	10-15%	Yes, unpurified culture
(Caron et al., 2016)	Pre-plating	1250x	N.R.	26 days	N.R.	N.R.
(Chal et al., 2016)	Pre-plating	N.R.	Yes	35 days	N.R.	N.R.
(Swartz et al., 2016)	No purification, differentiation analysed in original plate	N.R.	Yes	36 days	N.R.	N.R.
(Kim et al., 2017)	No purification, differentiation analysed in original plate	N.R.	N.R.	50 days	N.R.	Yes, unpurified culture
(van der Wal et al., 2017)	FACS	5 x 10^7x	Yes	35 days	60-80%	N.R.
This study	FACS	5 x 10^{11}x	Yes	35 days	20-97%	Yes, purified culture

N.R: Not Reported

Table S2. Optimization of CHIR99021 concentration

Control 1

CHIR99021	**Days**	**Confluency**	**PAX7$^+$ cells**
3 µM	4	65%	15-10%
3 µM	5	50%	7-10%
3 µM	8	40%	1-2%
3 µM	10	40%	0%
4 µM	4	85%	30-35%
4 µM	5	100%	35-40%
4 µM	8	75%	30-35%
4 µM	10	50%	5-10%
5 µM	4	60%	15-20%
5 µM	5	70%	20-25%
5 µM	8	0%	0%
5 µM	10	0%	0%

Control 2

CHIR99021	**Days**	**Confluency**	**PAX7$^+$ cells**
3 µM	4	95%	1-2%
3 µM	5	100%	2-3%
3 µM	8	90%	3-4%
3 µM	10	95%	3-4%
4 µM	4	95%	10-15%
4 µM	5	95%	9-12%
4 µM	8	95%	10-15%
4 µM	10	85%	1-2%
5 µM	4	95%	3-5%
5 µM	5	95%	15-20%
5 µM	8	50%	7-10%
5 µM	10	30%	0%

Table S3. RNA sequencing datasets used in this study

Data source	Accession	Abbreviation	Reference
ENA	ERR975347	Activated Muscle Stem Cell 2	(Charville et al., 2015)
ENA	ERR975349	Activated Muscle Stem Cell 1 P38 treated 2	(Charville et al., 2015)
ENA	ERR975346	Activated Muscle Stem Cell 1	(Charville et al., 2015)
ENA	ERR975348	Activated Muscle Stem Cell 1 P38 treated 1	(Charville et al., 2015)
NCBI	GEO: *GSM3024344*	MPCs control 1A	This study
NCBI	GEO: *GSM3024345*	MPCs control 1B	This study
NCBI	GEO: *GSM3024346*	MPCs control 2A	This study
NCBI	GEO: *GSM3024347*	MPCs control 2B	This study
ENA	ERR975345	Quiescent Muscle Stem Cell 2	(Charville et al., 2015)
ENA	ERR975344	Quiescent Muscle Stem Cell 1	(Charville et al., 2015)
NCBI	GEO: *GSM3024348*	MPCs 4 days differentiated control 1A	This study
NCBI	GEO: *GSM3024349*	MPCs 4 days differentiated control 1B	This study
NCBI	GEO: *GSM3024350*	MPCs 4 days differentiated control 2A	This study
NCBI	GEO: *GSM3024351*	MPCs 4 days differentiated control 2B	This study
NCBI	GEO: *GSM2452280*	Neural stem cell 1	(McGrath et al., 2017)
NCBI	GEO: *GSM2452281*	Neural stem cell 2	(McGrath et al., 2017)
NCBI	GEO: *GSM2452282*	Neural stem cell 3	(McGrath et al., 2017)
ENCODE	ENCBS476ENC	Dermal Fibroblast 1	N/A
ENCODE	ENCBS459ENC	Mesenchymal stem cell 2	N/A
ENCODE	ENCSR828TEI	Primary Myotube 1	N/A
ENCODE	ENCBS018ENC	Chrondocyte 1	N/A
ENCODE	ENCLB014ZZZ	Cardiomyocyte	N/A
ENCODE	ENCBS460ENC	Mesenchymal stem cell 1	N/A
ENCODE	ENCSR000CUI	Myosatellite cell 2	N/A
ENCODE	ENCSR000AAG	Smooth muscle cell	N/A
NCBI	SRX689200	Primary hepatocytes 2	(Kambara et al., 2014)
ENCODE	ENCSR000CUI	Myosatellite cell 1	N/A
ENCODE	ENCBS019ENC	Chrondocyte 2	N/A
ENCODE	ENCBS475ENC	Dermal Fibroblast 2	N/A
ENCODE	ENCBS945YXY	Primary Kidney epithelial cell 2	N/A
NCBI	SRX673854	Primary hepatocytes 1	(Kambara et al., 2014)
ENCODE	ENCBS007YZP	Primary Kidney epithelial cell 1	N/A
ENCODE	ENCSR828TEI	Primary Myotube 2	N/A
ENCODE	ENCSR444WHQ	Primary Myoblast 2	N/A
ENCODE	ENCBS293AAA	Embryonic stem cell 1	N/A

ENCODE	ENCBS624XJG	Embryonic stem cell 2	N/A
ENCODE	ENCSR444WHQ	Primary Myoblast 1	N/A
ENCODE	ENCBS485ENC	Hematopoietic stem cell	N/A

Table S4. Antibodies and primers used in experiments

Name	Dilution or Sequence 5'-3'	Company	Assay
Mouse-anti-MF20	1:50	DSHB	IF
Rabbit-anti-Myogenin	1:100	Santa Cruz (sc-576)	IF
Mouse-anti-PAX7	1:100 or 1:20	DSHB	IF or IHC
Mouse-anti-α-Actinin	1:100	Sigma-Aldrich (A7811)	IF
Mouse-anti-Myosin (fast)	1:100	Sigma-Aldrich (M4276)	IF
Mouse-anti-Titin	1:50	DSHB	IF
Rabbit-anti-Laminin	1:100	Sigma-Aldrich (L9393)	IHC
Mouse-anti-hSpectrin	1:100	Leica (SPEC1-CE)	IHC
Mouse-anti-hDystrophin	1:100	Millipore (MABT827)	IHC
Mouse-anti-hLaminA/C	1:100	Vector Laboratories (VP-L550)	IHC
GAA Exon 1-2 fw	AAACTGAGGCACGGAGCG	IDTDNA	RT-qPCR
GAA Exon 1-2 rv	GAGTGCAGCGGTTGCCAA	IDTDNA	RT-qPCR
Set_1_fw	TTCCCAGGGCCGGTTAATGT	IDTDNA	PCR
Set_1_rv	GCTCTGGGCGGAGGAATATG	IDTDNA	PCR
Set_2_fw	CCTGAGTCCGGACCACTTTG	IDTDNA	PCR
Set_2_rv	CACCGGTTCAATTGCCGAC	IDTDNA	PCR
Set_3_fw	GTCTCTCACTCGGAAGGACAT	IDTDNA	PCR
Set_3_rv	TACCCCGAAGAGTGAGTTTGC	IDTDNA	PCR

SUPPLEMENTARY METHODS

GAA enzyme activity assay

Differentiated myogenic progenitors were harvested with ice-cold protein lysis buffer (50 mM Tris (pH 7.5)), 100 mM NaCl, 50 mM NaF, 1% Triton X-100 and one tablet Protease Inhibitor Cocktail cOmplete, with EDTA, (Roche, Penzberg, Germany) for 10 minutes on ice. GAA enzyme activity was measured as described previously (Kroos et al., 2007). Total protein concentrations were determined with the BCA protein assay kit (Pierce, Thermo Scientific, Waltham, MA).

qRT-PCR

qRT-PCR was measured with a CFX96 real-time system (Bio-Rad, Hercules, CA). cDNA was diluted 5x or 10x times and 4 µL was used in a qRT-PCR reaction consisting of a total volume of 15 µL with 7.5 µL iTaq Universersal SYBR Green Supermix (Bio-Rad, Hercules, CA), 10 pmol/µL forward and reverse primers (Table S4). Per plate, a standard curve was included with 5 dilutions.

Immunofluorescent analysis of *in vitro* differentiation

Myogenic progenitors were stained as described previously (van der Wal et al., 2017). Briefly, cells were permeabilized for 5 minutes with 0.1% Triton X-100 (AppliChem, Darmstadt, Germany) in PBS and blocked for 30 minutes at room temperature in blocking solution (PBS-T (0.1% Tween, Sigma-Aldrich, Irvine, UK) with 3% BSA (Sigma-Aldrich, Irvine, UK)). Primary antibodies (Table S4) were incubated for 1 hour at room temperature and diluted into 0.1% BSA in PBS-T, washed with PBS-T and incubated with secondary antibodies (1:500, Alexa-Fluor-488-α-mouse, A11001, Alexa-Fluor-594-α-rabbit, A10474, Alexa-Fluor-488-α-rabbit,

A11008, Invitrogen, Carlsbad, CA; or horse anti-mouse biotin, BA-2000, Vector Laboratories, Burlingame, CA). When a secondary biotinylated antibody was used, cells were washed three times for 5 minutes with PBS-T and incubated with Streptavidine 594 (1:500, S-32356, Invitrogen, Carlsbad, CA,). The cells were subsequently washed two times for 5 minutes with PBS and incubated for 15 minutes with Hoechst (1:15000, Thermo Scientific, Waltham, MA) before imaging.

Generation of induced pluripotent stem cells
Control iPSC lines were previously reprogrammed, characterized and cultured as described in van der Wal et al. (van der Wal et al., 2017). Healthy control 3 and healthy control 4 iPSCs were a gift from Dr. Mehrnaz and Prof. Joost Gribnau . Healthy control 2 (previously characterized in (Dambrot et al., 2013)), healthy control 5 (LUMC0004iCTRL10), and healthy control 8 (LUMC0030iCTRL12) iPSCs were gifts from Dr. Christian Freund and Prof. Christine Mummery. Using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Walkersville, MD), the iPSC lines were regularly tested for contamination with mycoplasma. All results in this study were obtained with cultures that had tested negative. The identities of cell lines used in this study were confirmed by DNA sequencing.

Generation and expansion of myogenic progenitors from iPSCs
iPSC cultures in 100 mm dishes were used to initiate myogenic differentiation as described previously (van der Wal et al., 2017). Briefly, after 5 days of iPSC expansion, differentiation into myogenic progenitors was started with myogenic differentiation medium (DMEM/F12, 1% Insulin-Transferrin-Selenium-Ethanolamine (ITS-X), 1% penicillin/streptomycin/L-glutamine (P/S/G), all Gibco, Waltham, MA) supplemented with 3.5 μM CHIR99021 (Axon Medchem, Groningen, the Netherlands) for 5 days; and changed to myogenic differentiation medium supplemented with 20 ng/ml FGF2 (Prepotech, Rocky Hill, NJ) for 14 days. For the last 16 days, cells were cultured in myogenic differentiation medium only. Myogenic progenitors were purified using FACS with anti-C-MET-APC (1:50, R&D systems, Minneapolis MN), and anti-HNK-1-FITC (1:100, Aviv Systems Biology, San Diego, CA) antibodies; and Hoechst (33258, Life Technologies, Carlsbad, CA) was added to stain live cells. The c-MET^{+}/Hoechst^{+}/Hnk-1^{-} fraction was sorted in myogenic progenitor proliferation (MMP) medium (DMEM high-glucose supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin/L-glutamine and 100 ng/ml FGF2) supplemented with 1x Revitacell supplement (Gibco, Waltham, MA) on ECM (Sigma-Aldrich, E6909)-coated dishes as described (van der Wal et al., 2017).

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Chapter 5

Generation of human iPSC-Derived Myotubes to Investigate RNA-Based Therapies *In Vitro*

Generation of human iPSC-Derived Myotubes to Investigate RNA-Based Therapies *In Vitro*

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Running head:

Human iPSC-derived skeletal muscle for *in vitro* testing

Methods Molecular Biology, in press

Summary/Abstract

Alternative pre-mRNA splicing can be cell-type specific and results in the generation of different protein isoforms from a single gene. Deregulation of canonical pre-mRNA splicing by disease-associated variants can result in genetic disorders. Antisense oligonucleotides (AONs) offer an attractive solution to modulate endogenous gene expression through alteration of pre-mRNA splicing events. Relevant *in vitro* models are crucial for appropriate evaluation of splicing modifying drugs. In this chapter, we describe how to investigate the splicing modulating activity of AONs in an *in vitro* skeletal muscle model, applied to Pompe disease. We also provide a detailed description of methods to visualize and analyse gene expression in differentiated skeletal muscle cell for the analysis of muscle differentiation and splicing outcome. The methodology described here is relevant to develop treatment options using AONs for other genetic muscle diseases as well, including Duchenne muscular dystrophy, myotonic dystrophy, and facioscapulohumeral muscular dystrophy.

Key words

Splicing, human iPSC, skeletal muscle, antisense oligonucleotides, *in vitro* models

1. Introduction

Pre-mRNA splicing is a highly conserved mechanism in eukaryotes that plays a role in pre-mRNA processing. Alternative splicing can diversify gene function to produce isoforms with specific functions in distinct cell types [1,2]. Human genetic variations can lead to defects in pre-mRNA splicing that cause human disease [3]. Modulation of pre-mRNA splicing can be directed to correct aberrant splicing, to skip protein coding variants, to restore the reading frame, or to prevent expression of toxic gene products. This is possible by targeting antisense oligonucleotides (AONs) towards canonical splice sites or to *cis*-acting regulatory elements such as cryptic splice sites or splicing silencers/enhancers [4]. Alternative splicing in skeletal muscle is abundant and essential for muscle development and function [5]. Deregulation of pre-mRNA splicing in skeletal muscle is known to be the underlying cause of multiple human myopathies [5]. Suitable *in vitro* and *in vivo* models are crucial to investigate novel splicing modulating drugs in target cells and tissues.

In vitro human skeletal muscle models can be obtained directly from muscle biopsies, or these can be generated by (trans-)differentiation of primary fibroblasts, pluripotent stem cells or non-muscle cells with myogenic capacity like pericytes and mesoangioblasts [6-8]. Several protocols have been described to generate muscle progenitor cells (MPCs) derived from human patient-derived induced pluripotent stem cells (hiPSCs) using directed differentiation methods for disease modelling [9-11].

Here we describe how purified, expandable hiPSC-derived MPCs, generated using a transgene-free procedure [11] can be differentiated into multinucleated myotubes to test the modulating activity of AONs. These methods can be used to analyse splicing correction *in vitro* to develop RNA-based therapies for muscle disorders. We have used this strategy to test AONs for Pompe disease [12] and describe the methodology here in detail.

2. Materials

All cell culture work needs to be performed under sterile conditions in safety cabinets. All cell lines should be tested for mycoplasma following the manufacturer instructions (Lonza; LT07-318). Cell lines are cultured at 5% CO_2 and 37°C in humidified incubators.

2.1 Skeletal Muscle Progenitor Cell Culture

1. Human MPC lines (*See* Note **1**)
2. DMEM 4,5 g/L Glucose
3. Foetal Bovine Serum
4. Penicillin/Streptomycin/Glutamine 100X (p/s/g)
5. Fibroblast Growth Factor 2 (FGF2) (*See* **Note 2**)
6. Sterile cell culture grade Bovine Serum Albumin (7,5% BSA)
7. TrypLE™ Express Enzyme (1X), phenol red
8. Phosphate Buffered Saline (DPBS)
9. Extracellular Matrix gel from Engelbreth (ECM; 1X)
10. DMEM:F12
11. Insulin/Transferrin/Selenium 100X
12. DMSO
13. Freezing containers

2.2 Cell Culture media

1. Proliferation medium: DMEM 4,5 g/L Glucose, supplemented with 10% FBS, 1X Pen/Strep and 100 ng/ml FGF2 (added directly to plate/well).
2. Differentiation medium: DMEM:F12, supplemented with 1X ITS-X and 1X Pen/Strep.

2.3 Antisense Oligonucleotide design and delivery

1. Phosphorodiamidate morpholino oligomer (PMO) AONs , LLC.
2. Endoporter , LLC
3. MilliQ filtered sterile water

2.4 Immunofluorescence

1. 4% Paraformaldehyde (diluted from a 32% solution in PBS)
2. 0,1% Tween (diluted in PBS from a 100% solution)
3. 0,3% Triton-X100 (diluted in PBS from a 100% solution)
4. Bovine Serum Albumin (BSA)
5. Primary antibodies: Mouse-α-MYH1E (1:50, MF20 supernatant, DSHB), Rabbit-α-MYOGENIN (1:100, sc-576, Santa Cruz), Rabbit-α-MYOD (1:100, sc-304, Santa Cruz), Mouse-α-PAX7 (1:100, concentrate, DSHB)
6. Secondary antibodies: Horse-α-mouse biotin (1:250, Vector Laboratories), Alexa Fluor-594-a-goat, Alexa Fluor-488-α-mouse, Alexa Fluor-594-α-rabbit, Alexa Fluor-488-α-rabbit (1:500, Invitrogen)
7. Tertiary: Streptavidin 594 (1:500, Invitrogen, S-32356)
8. Hoechst 33342 (1:15000, Invitrogen, H3570)
9. Nikon wide field microscope (10X and 20X objectives).

2.5 RNA isolation, cDNA synthesis and quantitative RT-PCR (RT-qPCR)

1. RNA isolation kit
2. cDNA Synthesis kit
3. iTaq Universal SYBR Green Supermix
4. Hard-Shell 96-Well PCR Plates
5. Thermocycler
6. Real-time thermocycler
7. Spectrophotometer
8. Agarose
9. Ethidium Bromide
10. Primers (*See* Table 1)

3. Methods

3.1 Expansion, Cryopreservation and Differentiation of MPCs

3.1.1 Expansion

1. MPCs grow optimally when the confluency is between 30% and 90%. It is important to maintain this cell density throughout the expansion (*See* **Note 3**).
2. Plate cells onto ECM coated plates (*See* **Note 4**). Coat plates using a solution of ECM (1:200) diluted in Proliferation medium without FGF2. Coating solution is left on the plates for 30 min at RT.
3. For cell detachment, first wash plates in pre-warmed PBS at 37ºC and then treat the cells with a 1:1 pre-warmed solution of TrypLE™ Express Enzyme and PBS (3ml for 10cm plates) for 3-5 min at 37ºC.
4. Collect cells using 5 volumes of Proliferation medium and centrifuge for 4 min at 200 g.
5. Resuspend cells using Proliferation medium, transfer to pre-coated plates (remove coating solution, do not wash) and add 100 ng/ml of FGF2 directly into the plate (*See* **Note 5**).
6. Immediately transfer plates to a humidified incubator and perform cross movements to ensure appropriate cell spreading and mixing of FGF2.

3.1.2 Freeze-thaw

1. Thaw vials of MPCs in a pre-warmed water bath, transfer cell suspension slowly into 5 volumes of Proliferation medium (no FGF2) and centrifuge 4 min at 200 g.
2. Plate cells in pre-coated plates using Proliferation medium plus 100 ng/ml of FGF2 freshly added to the cells.
3. Freeze cells using Proliferation medium (plus 100 ng/ml FGF2) and 10% DMSO in 1 ml cryovials and store in freezing containers at -80ºC for 24h (at least) prior to long-term storage in liquid nitrogen tanks.

3.1.3 Differentiation into Multinucleated Myotubes

1. Grow cells to reach >90% confluency (avoid 100% confluency) and then switch to Differentiation medium for 4 days without refreshing (*See* **Note 6**). Wide field images of differentiated myotubes are shown in Figure 1.

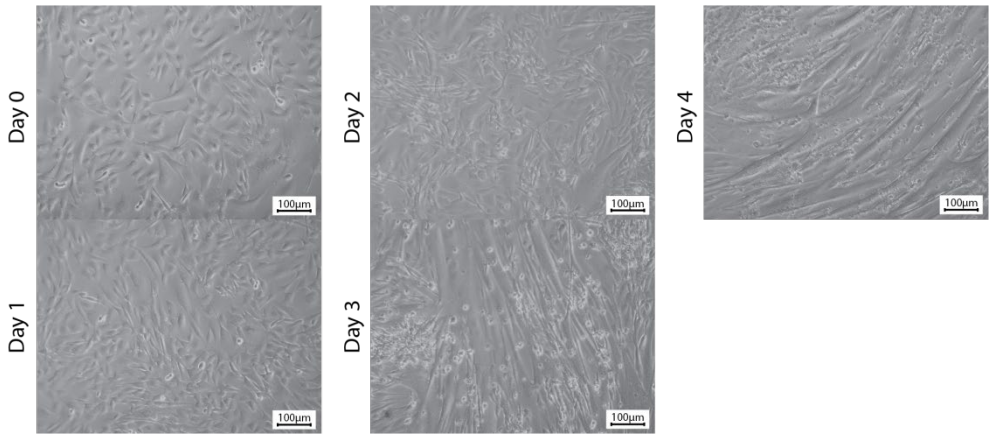

Figure 1. Wide field images of differentiating MPCs. *Representative images of the differentiation of MPCs over 4 days. Scale bar 100 µm.*

3.2 Delivery and Efficacy of Antisense Oligonucleotides in Patient-derived Myotubes

3.2.1 Transfection

1. Resuspend the PMO AONs in RNAse free MilliQ at a concentration of 1 mM.
2. Add 4,5 µl of Endoporter reagent per ml of medium directly to the cells and mix by gentle shaking (*See* **Note 7**).
3. Add the desired amount of PMO AONs to the cells and mix by gentle shaking.
4. Transfect AONs 1 day prior differentiation (day -1). Cells should be 60%-80% confluent.
5. Switch to differentiation medium (day 0).
6. Leave cells to differentiate for 4 days and either collect protein or RNA, or fix cells for immunofluorescence.

3.2.2 Immunofluorescence

1. For immunofluorescence analysis of patient-derived myotubes, prepare cells using 48 well plates.
2. Wash cells once in PBS.
3. Fix cells using 4% PFA in PBS for 10 min at RT, remove and add PBS. Cells can be stored at 4ºC before proceeding.
4. Wash twice in PBS for 2 min each.
5. Incubate for 10 min with 0,3% Triton-X100 in PBS for permeabilization.
6. Incubate for 30 min with 3% BSA, 0,1% Tween in PBS for blocking.
7. Repeat washing step 4.
8. Incubate with primary antibodies for 1 hour at RT in 0,1% BSA, 0,1% Tween in PBS (*see* **Note 8**).
9. Repeat washing step 4.
10. Incubate with secondary antibodies for 45 min at RT in 0,1% BSA, 0,1% Tween in PBS.
11. Repeat washing step 4.
12. If biotinylated antibodies were used, incubate with tertiary for 30 min at RT in 0,1% BSA, 0,1% Tween in PBS.
13. Repeat washing step 4.
14. Counterstain with Hoechst nuclear staining (1:15000) in PBS for 10 min.
15. Remove and add PBS.
16. Take images of five random fields with 10x or 20x lens in MYH1E stained myotubes (*See* Figure 2) to calculate the fusion index. The fusion index is determined as the percentage of nuclei present in multinucleated MYH1E positive cells (>2 nuclei in one cell) with respect to the total number of nuclei.

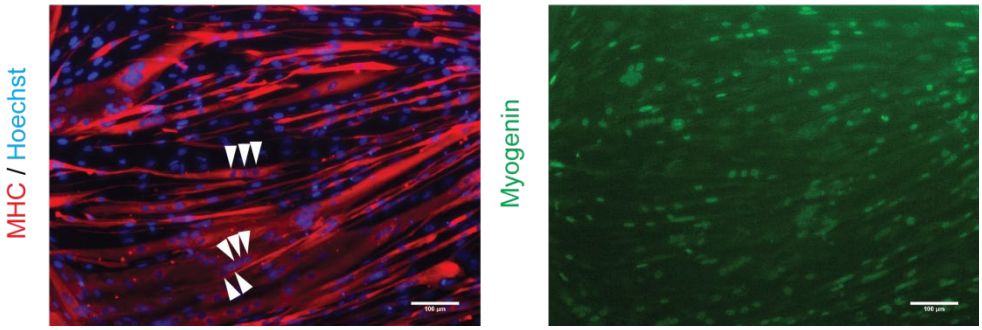

Figure 2. Immunofluorescence images of 4 days differentiated MPCs. *MPCs were stained with MYH1E (red), MYOGENIN (green) and the nuclei with Hoechst (blue). Arrowheads indicate nuclei present in multinucleated myotubes. Scale bar 100 µm.*

3.2.3 RT-qPCR

1. Harvest RNA after 4 days of differentiation using 350 µl of the lysis buffer (or the amount indicated in the first step of the RNA isolation kit) per well of a 12 well-plate.
2. Purify RNA following the instructions of the preferred RNA isolation kit.
3. Retrotranscribe 300-500 ng of total RNA into cDNA using a cDNA Synthesis Kit.
4. Dilute cDNA samples 10X and prepare the qPCR reaction using iTaq Universal SYBR Green Supermix.
5. Amplify the cDNA of interest using a Real Time System.
6. To analyse alternative splice variants in patient-derived myotubes, we normalize gene expression using each of the following four genes: *MYOD*, *MYOG (Myogenin)*, *LAMP1* and *LAMP2* (See **Note 9**). Gene expression is calculated using the ΔCt method for each housekeeping gene. Thereafter, the average value of the four normalized expression values is calculated.

4. Notes

1. Here we only used transgene-free derived muscle progenitor cells from hiPSCs as described in [11]. However, we anticipate that other sources of myogenic cells would also be applicable.

2. The FGF2 stock powder is dissolved in 0,1% BSA (Sterile cell culture-grade BSA diluted in PBS and filtered using a 0,22 µm filter) and aliquoted using tips and tubes that were coated with 0,2% BSA in PBS. The dissolved FGF2 can be stored at -80ºC. Each aliquot is used for maximally 1 week after thawing (kept at 4ºC) and 100 ng/ml is added directly to the cell culture medium every 2 days. When adding FGF2 every 2 days, this can be done without refreshing cell culture media. However, cell culture media must be refreshed every 3 days.
3. MPCs spontaneously differentiate at a confluency of >90% and loose proliferative capacity in culture.
4. Here we only used ECM for our studies. However, we anticipate that other coating materials can be used as well.
5. We typically plate a 1/4 or 1/6 dilution of cells to get a 60-90% confluency in 2 days and 3 days respectively using the same plate surface area.
6. There are different methods to differentiate MPCs into multinucleated myotubes [9,10,12,11]. For these studies we used DMEM:F12, 1x ITS-X, 1X p/s/g for 4 days without refreshing. Longer differentiation periods might result in cell detachment due to spontaneous contraction.
7. Endoporter reagent is specifically designed for transfection of PMO AONs and was used by us in the following studies [4,11]. Endoporter reagent does not form a complex with AONs but it enhances endocytosis in cells. The amount of Endoporter used is independent of the concentration of PMO AONs. Other backbones might require different delivery reagents.
8. The following proteins are commonly used to assess myogenic potential of differentiating MPCs across species: Myosin heavy chain (MYH1E, cytoplasmic), Myogenin (MYOG, nuclear) and MYOD (nuclear). PAX7 (nuclear) can be used to identify the muscle stem cell fraction.
9. To normalize gene expression, we observed that the following genes involved in myogenesis: *MYOD* and *MYOG*; and the following involved in lysosome biogenesis: *LAMP1* and *LAMP2*; do not change expression levels among patient and healthy donor derived myotubes. We used these genes in this study [12].

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Table 1. Primers used for RT-qPCR.

Primer Target	**Sequence (5’ to 3’)**
MYOD fw	CACTCCGGTCCCAAATGTAG
MYOD rv	TTCCCTGTAGCACCACACAC
MYOG fw	CACTCCCTCACCTCCATCGT
MYOG rv	CATCTGGGAAGGCCACAGA
LAMP1 fw	GTGTTAGTGGCACCCAGGTC
LAMP1 rv	GGAAGGCCTGTCTTGTTCAC
LAMP2 fw	CCTGGATTGCGAATTTTACC
LAMP2 rv	ATGGAATTCTGATGGCCAAA

Chapter 6

Shortening of intron 1 restores the c.-32-13T>G *GAA* splicing variant in iPSCs derived skeletal muscle cells from childhood/adult Pompe patients

Shortening of intron 1 restores the c.-32-13T>G *GAA* splicing variant in iPSCs-derived skeletal muscle cells from childhood/adult Pompe patients

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Submitted

ABSTRACT

Pompe disease is a lysosomal storage disorder with progressive skeletal muscle wasting caused by deficiency of the enzyme acid alpha glucosidase (GAA). The common c.-32-13T>G (IVS1) splicing variant is characterized by aberrant splicing, leading to slow but progressive accumulation of glycogen in the skeletal muscles of late onset Pompe patients. Here, we partially deleted the intron 1 of the *GAA* gene using CRISPR/Cas9 in IVS1 patient derived iPSCs. Differentiating iPSC-derived skeletal muscle cells restored *GAA* expression and enzyme activity comparable to healthy individuals from just a single targeted allele. Using engineered patient derived skeletal muscle cells as isogenic controls, we identified distinct transcriptomic profiles linked to GAA enzyme activity during muscle differentiation. Gene expression changes were associated with increased myogenic progression, aerobic glycolysis and glycogen synthesis in classic-infantile derived cells. Skeletal muscle cells derived from late onset patients resulted in reduced expression of genes involved in glucose import and anaerobic glycolysis. These results illustrate the capacity of using isogenic controls to uncover subtle gene expression changes in progressive skeletal muscle disorders. Additionally, we present a novel gene-editing strategy for the permanent correction of aberrant GAA expression in affected skeletal muscles of IVS1 Pompe disease patients.

Keywords

Pompe disease; IVS1; gene-editing; isogenic iPSCs; engineered skeletal muscle; disease modeling

INTRODUCTION

Pompe disease is an autosomal recessive lysosomal storage disorder that is characterized by skeletal muscle wasting, and is caused by variants in the acid alpha glucosidase (*GAA*) gene[1]. Classic-infantile (CI) Pompe patients have a complete lack of GAA enzyme activity and present shortly after birth with a hypertrophic cardiomyopathy and progressive generalized skeletal muscle weakness[1–3]. The childhood and adult onset forms of Pompe disease (hereinafter named late onset, LO) mostly present later between birth and 60 years of age and express 1%-20% of residual GAA activity[4]. In childhood and adult Pompe disease, 90% of the patients in the Caucasian population harbor the common c.32-13T>G (IVS1) variant on one allele that results in aberrant splicing of exon 2, leading to ~10-15% of residual GAA enzyme activity[5–8].

To date, enzyme replacement therapy (ERT) is the only available treatment for Pompe disease[9,10]. ERT is life saving for classic-infantile and childhood/adult Pompe disease patients. However, the variation in treatment outcome, the high dose and the high costs encourage researchers to improve the understanding of skeletal muscle disease and search for alternative and next generation treatment options[11–18]. Most research on Pompe disease is performed using models that completely lack GAA enzyme activity, representing the classic-infantile form[19]. Knock-out mouse models have been essential to uncover fundamental pathophysiological mechanisms of Pompe disease. However, significant species-specific differences exist in many aspects of metabolism and (alternative) splicing, which could complicate further mechanistic insight[20,21].

We recently reported the generation of a transgene-free myogenic differentiation procedure that generates pure myogenic progenitor cell (MPC) cultures with large expansion rates[18]. These MPCs showed the capacity to differentiate into multinucleated skeletal muscle cells that are suitable for quantitative analysis and high-throughput screening. We applied this system to iPSCs from childhood/adult onset Pompe disease that carried the IVS1 variant, which revealed a complete rescue of aberrant splicing by antisense oligonucleotides (AONs)[22]. AON-mediated therapy could provide a temporary rescue and will require repeated administration. Ultimately, genome editing

techniques could provide permanent correction of genetic defects and potentially ameliorate the disease pathology[23,24]. A pioneering study from Raben et al, demonstrated that removing a large portion of the intron 1 from the *GAA* gene, both wild type or with the IVS1 variant, resulted in increased expression of *GAA* in a minigene construct[25]. It remained to be determined whether the partial removal of intron 1 in genomic DNA is also able to enhance *GAA* expression in IVS1 Pompe patients. Differences in lysosomal glycogen content are difficult to detect in cells derived from patients with LO Pompe disease, likely reflecting the slow progression of the disease. In some patients, development of symptoms does not occur before 60 years of age. We hypothesized that more subtle changes may be detectable before the aberrant lysosomal pathology characteristic of Pompe disease becomes evident. By restoring GAA expression in patient skeletal muscle cells, we generated an isogenic model supportive for the detection of gene expression changes with minimum genetic background variation.

In this study, we first deleted part of intron 1 in a minigene system carrying the IVS1 variant. This led not only to correction of aberrant splicing of *GAA* but also increased the expression of GAA enzyme activity. We then deleted a large part of intron 1 at the genomic level using CRISPR/Cas9 in patient-derived iPSCs carrying the IVS1 variant on one allele, and a fully deleterious *GAA* variant on the other allele. Differentiation of gene-corrected iPSCs into skeletal muscle cells resulted in increased expression of *GAA* and correction of aberrant splicing characteristically observed in patients with the IVS1 *GAA* variant. This resulted in GAA enzyme activity comparable to the activity found in cells of individuals with two wild type alleles. We previously genetically corrected iPSCs derived from a patients with CI Pompe disease in which we introduced a WT cDNA copy of the *GAA* gene in the *AAVS1* safe harbor[18]. This resulted in correction of GAA deficiency and lysosomal glycogen clearance when compared to genetically matched controls carrying an empty transgene. We finally applied genome wide RNA-sequencing to generate a transcriptomic profiling of isogenic skeletal muscle cells derived from CI and LO Pompe patients. This led to the identification of large changes in gene expression in differentiating myotubes. Particularly, alteration in glucose/glycogen metabolism and muscle contractile properties and development, suggesting that these

processes are responsive to deficiency and restoration of lysosomal GAA enzyme activity. These results demonstrate a novel gene correction strategy for the IVS1 variant in Pompe disease, and provide evidence for a molecular link between GAA, skeletal muscle development and glucose metabolism.

RESULTS

Shortening of GAA intron 1 in a minigene increases expression and corrects splicing of GAA in an IVS1 background

The IVS1 (c.-32-13T>G) variant in Pompe disease is located in the polypyrimidine-tract (pY-tract) of intron 1 of the *GAA* pre-mRNA, and causes aberrant splicing. This leads to several additional splice variants such as SV2 and SV3 that do not include the AUG translation start site (figure 1A). Roughly ~10% of the transcripts are correctly spliced, which explains the residual activity measured in cells of patients and its association with a childhood/adult onset Pompe disease phenotype[5–7]. Previous removal of a large part of intron 1 (2kb) in a minigene, that spanned *GAA* exon 1 to exon 3, resulted in increased mRNA expression, both in an IVS1 and a wild type (WT) background[25]. This was explained by the removal of a binding site for the transcriptional repressor HES-1[26,27]. However, it was unknown to what extent shortening of intron 1 might affect GAA splicing. To test this, we used a similar minigene that has been described by us previously[22], and used this to delete 1.9 kb of intron 1 (figure 1B, C). HEK293T cells were transfected with this newly generated minigene and we analyzed the construct expression by flanking exon RT-PCR (figure 1D). Compared to the WT minigene, the uncut IVS1 minigene showed decreased expression of the normal transcript N, while expression of SV2 and SV3 were increased. After the removal of 1.9 kb of intron 1, from both the WT and the original IVS1 minigene a higher expression of normal transcript was detected both for the WT deleted intron 1 (∆i1) and IVS1 ∆i1 minigenes. To exclude that the elevated expression was the result of changes in transfection efficiency, *Neomycin* was amplified and showed no significant differences in expression (figure S1). To quantify the expression levels, RT-qPCR was applied with specific primers targeting the N, SV2 and SV3 transcripts[22] (figure 1E). Intron 1 deletion of the WT minigene caused a 38 fold increase in expression of the N variant, while

expression of SV2 and SV3 transcripts were increased 4 and 8 fold, respectively. When comparing IVS1 with IVS1 ∆i1 minigenes, intron 1 deletion caused a 94 fold increase in expression for product N, and a 13 and 23 fold increased expression for the SV2 and SV3 variants, respectively. These results suggested that: 1) intron 1 deletion increases expression of normal *GAA* transcript, and 2) in addition to this, intron 1 deletion facilitates normal *GAA* splicing. The latter conclusion can de deduced from the observation that intron 1 deletion induced a larger increase in expression of the SV2 and SV3 in the IVS1 minigene compared to the WT minigene. Taken together, 1.9kb removal of intron 1 increased expression of normal and alternative variants in *GAA* minigenes.

Shortening of the genomic intron 1 sequence in patient-derived induced pluripotent stem cells (iPSCs)

We next wished to investigate the efficiency to remove part of intron 1 on the IVS1 allele in patient lines using CRISPR/Cas9. To this end, we reprogrammed 2 fibroblast cell lines from different Pompe patients that carried the IVS1 and c.525del variant into iPSCs. A lentiviral vector carrying the four reprogramming factors (Oct4, Sox2, KLF4 and C-myc) was used[28]. The deleterious c.525del variant causes degradation of *GAA* mRNA by a reading frame shift, rendering transcription from the IVS1 allele as the only source of *GAA* mRNA, thereby reducing background[29]. Two single guide RNA (sgRNA) sequences were selected to delete 2.1 kb of intron 1 using the CRISPRscan algorithm[30], and these showed low predicted numbers of off-target hits (<1) (note the IVS1 variant remain in the ∆i1 product) (figure 2A). PCR screening of iPSC colonies was performed with primers that span the deletion and amplified the full-length (WT) product and the 2.1 kb ∆ intron 1 product. To obtain a pure clone, single colonies were picked and screened using this PCR strategy for two patient lines (figure 2B). For each patient iPSC line, we selected clones that contained a heterozygous deletion and performed a second PCR and sequencing reaction to determine whether the deletion was present at the IVS1 allele (figure 2D). Analysis of the cleavage site of the selected clones showed that both patient iPSC clones contained a deletion at the IVS1 allele with a removal of 2137 bp (figure 2F). These sequencing reactions

however, were not ideal to determine the editing efficiency at the IVS1 allele. Hence, to quantify the efficacy of the gene-editing outcome, we repeated the same

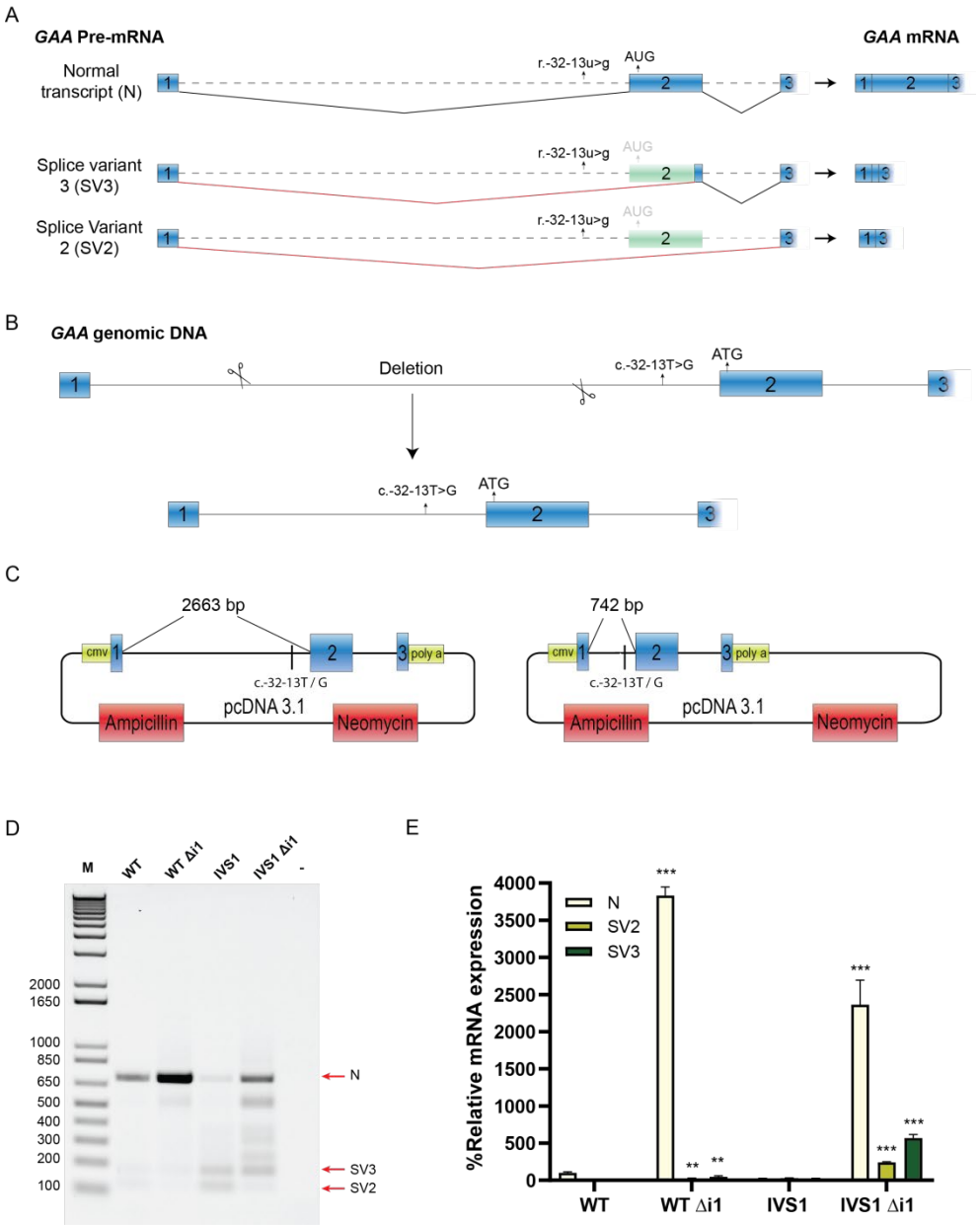


Figure 1. Partial removal of 1921 bp in IVS1 and WT GAA minigenes increases expression and corrects splicing. *(A) Outline of the effect of the IVS1 variant (c.-32-13T>G) on aberrant splicing of exon 2 of GAA. (B) Cartoon of removal of a large part of intron 1 of GAA in genomic DNA. (C) Cartoon*

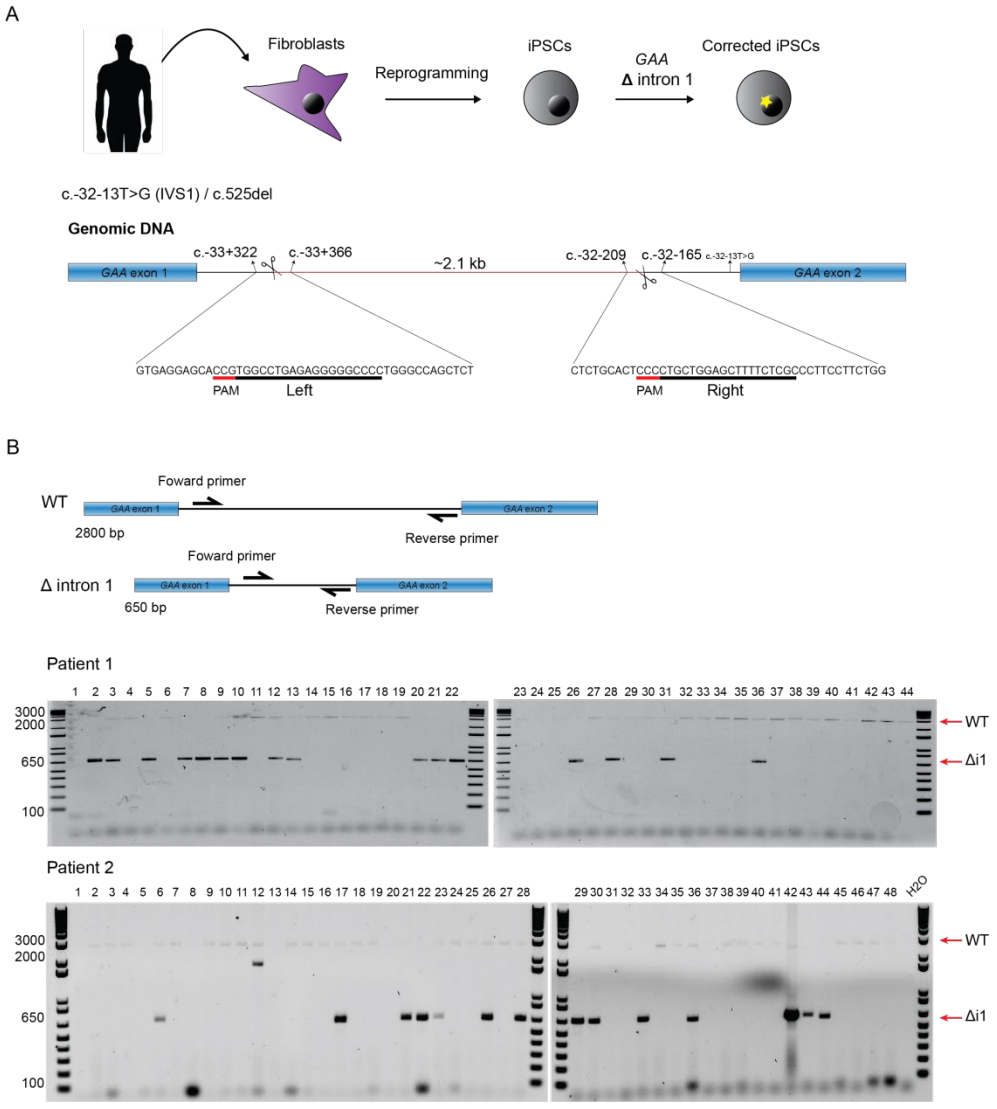
*of the GAA minigene in the pcDNA3.1 vector containing a CMV promoter (yellow), exon 1, exon 2, exon 3 (blue) and a polyA sequence (yellow). The IVS1 variant was introduced in intron 1 and 1921 bp was removed. (D) Transfection of the minigene into HEK293T cells and RT-PCR on cDNA with primers spanning exon 1 – exon 3. Red arrows indicate the normal transcript (N), splice variant 3 (SV3) and splice variant 2 (SV2). (E) RT-qPCR with specific primers against N, SV2 and SV3. mRNA expression was calculated using the deltaCT method. Data are means ± standard deviation of three biological replicates. * p < 0.05, **p <0.01 and ***p < 0.001.*

gene-editing strategy and used two on-target PCR assays on one patient line (Patient 1) (figure 2C). 1) Using primers that span from exon 1 to exon 3. With this first PCR, we could determine which colonies were successfully gene-edited and which ones were gene-edited at one or both alleles. 2) Taking the heterozygous gene edited colonies, we used IVS1 allele specific primers spanning from the exon 1 to the IVS1 variant. With this second PCR we could determine which colonies contained the deletion at the IVS1 allele. Results demonstrated that from 62 iPSC colonies successfully analyzed, 8.06% showed Δ intron 1 at the IVS1 allele, 11.29% at the 525del allele, 19.35% at both alleles and 61.29% were not gene-edited (figure 2E). This gene editing approach could efficiently generate multiple clones from different patient lines with a deletion of 2.1 kb at the intron 1 on the IVS1 allele. The editing outcome was successfully determined using different on-target PCR reactions and resulted in high gene-editing efficiency of patient iPSCs without neither antibiotic resistance nor reporter selection. We further validated selected clones via sequencing and expanded isolated clones carrying the Δ intron 1 at the IVS1 allele.

Shortening of GAA intron 1 increases GAA expression and corrects exon 2 splicing in iPSC derived skeletal muscle cells

Alternative pre-mRNA splicing is a highly conserved and complex mechanism in eukaryotes and it is not clear whether the shortening of intron 1 at the endogenous *GAA* loci could also influence GAA expression. Hence, we decided to study whether the expression of endogenous *GAA* could also have been altered by the removal of 2.1 kb of intron 1 on the IVS1 allele as expected from the minigenes. In addition, alternative pre-mRNA splicing is cell-type specific, and patients suffering from late onset Pompe disease show severe progressive disease symptoms in the skeletal muscle. We generated myogenic progenitor cells (MPCs) derived from

both LO isogenic iPSCs investigated altered GAA expression. To generate skeletal muscle cells, iPSC lines were differentiated using a transgene free differentiation procedure as described by us previously[18]. After expansion of iPSCs, myogenic differentiation was initiated and followed by purification of MPCs after 35 days using fluorescent activated cell sorting (figure 3A). Differentiation efficiency of patient 1 and 2 iPSCs into MPCs was not changed after shortening of intron 1 (d



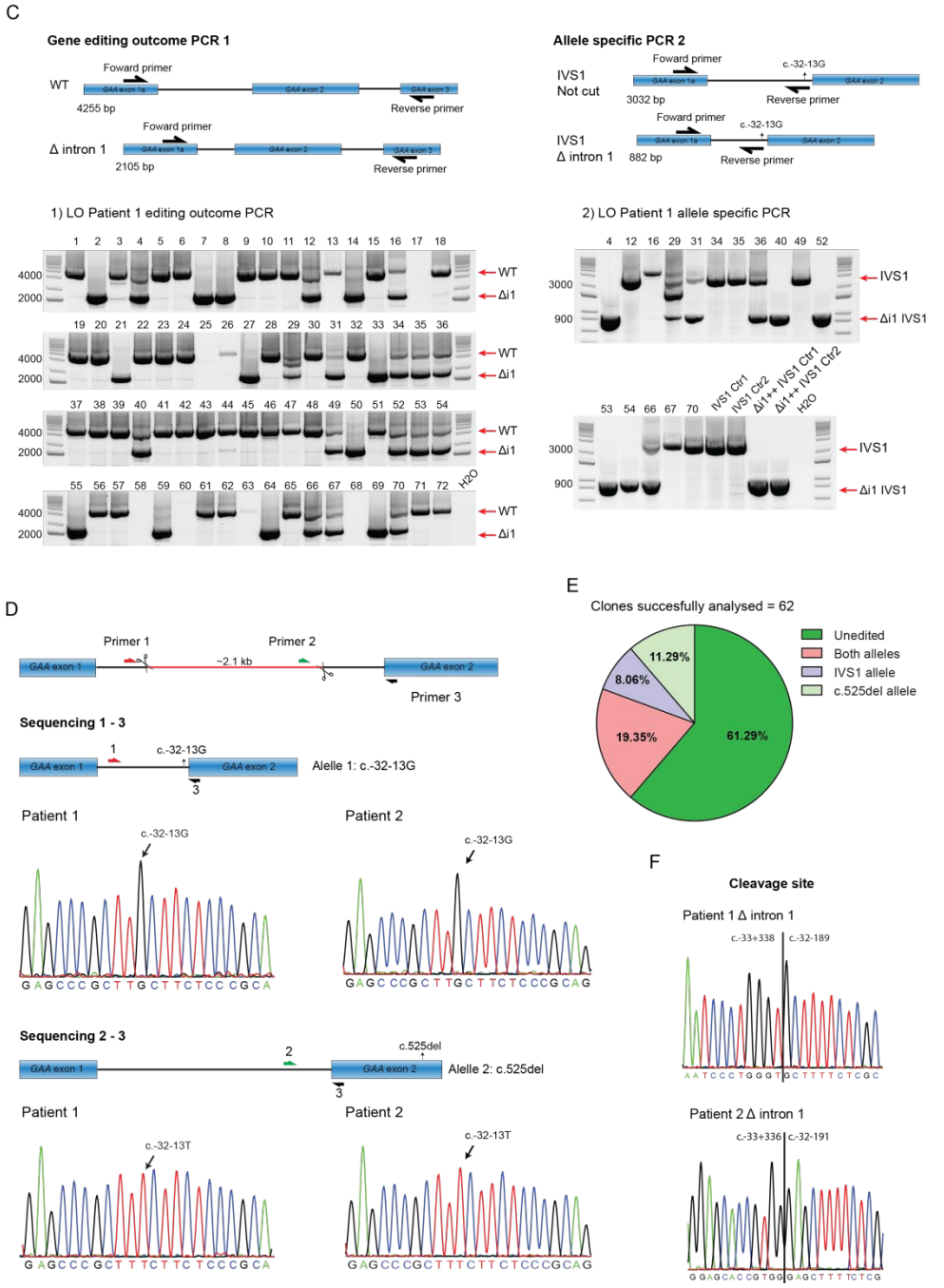


Figure 2. Removal of 2.1 kb in intron 1 of GAA using CRISPR/Cas9 in patient-derived induced pluripotent stem cells. *(A) Cartoon showing the reprogramming of human fibroblasts carrying the IVS1 variant (c.-32-13T>G) and the c.525del into induced pluripotent stem cells (iPSCs), followed by gene*

editing using CRISPR/Cas9. Two single guide RNA (sgRNA) sequences targeting intron 1 on the genomic sequence are indicated with the black bar. The red bar represents the PAM site. (B) RT-PCR from single iPSC colonies from LO patient 1 and LO patient 2, nucleofected with CRISPR/Cas9. (C) PCR strategy used to determine the editing efficiency using patient 1 iPSCs. Left: PCR 1 reaction amplifying wild type (WT) and ∆ intron 1 containing product. Right: PCR 2 reaction amplifying only the IVS1 allele. (D) Sequencing of selected iPSC colonies from B as indicated in the cartoon to amplify the product containing the partial deletion of intron 1 (sequencing 1 – 3: primer 1 with primer 3) and the uncut wild type product (sequencing 2 – 3: primer 2 with primer 3). (E) Pie chart showing the results of the editing efficiency among 62 iPSC clones successfully analyzed from C. (F) Sequence analysis of PCR products spanning the cleavage site from the selected iPSC clones from D.

(data not shown). Isogenic MPCs were amenable to expand and further differentiate into multinucleated myotubes for 4 extra days (figure 3B). *GAA* splicing analysis using flanking exon RT-PCR of exon 2 showed that expression of the splice variant N was enhanced while the aberrant SV2 and SV3 products were almost undetectable in the ∆ intron 1 myotubes (figure 3C). This correction was similar to what observed in 4 different control myotubes derived from healthy individuals. We could also detect a light band on top of the N product on patient 1 ∆ intron 1 which almost disappears after slowly cooling the RT-PCR products (figure S2A). This could be due to folding of the RT-PCR products. The splicing product-specific qRT-PCR confirmed the results depicted on figure 3C. When comparing WT with ∆ intron 1 cells from patient 1 and 2, a 4.7 and 3.7 fold increased expression for product N, and a 17.4 and 15.5 fold reduction in expression for product SV2 was observed respectively (figure 3D). Reduction of SV3 expression in cells from patient 1 and 2 was 8.3 and a 12.8 fold, respectively. These results only partially mimicked the results obtained using the minigene: whereas all splice products showed increased expression upon intron 1 deletion in the minigene, although to different extents, only the N product was increased while SV2 and SV3 splice products were strongly reduced following intron 1 deletion at the genomic level. This indicated that intron 1 deletion corrected canonical *GAA* pre-mRNA splicing of the IVS1 variant. We finally analyzed if this increased gene expression of *GAA* could also result in higher enzymatic activity. Not only GAA activity was increased 5.5 and 8.5 fold in patient 1 and 2 respectively, but also this increase reached similar levels to what we found in healthy controls (figure 3D, S2B). The removal of 2.1 kb of intron 1 on the IVS1 allele in patient skeletal myotubes restored the levels of WT GAA. These levels were found similar to what observed

in healthy controls and indicate a correction of *GAA* pre-mRNA splicing and enhanced expression of endogenous *GAA* from a single allele.

Large transcriptional changes occur early in response to GAA deficiency in differentiating Pompe patient derived skeletal muscle cells

In our previous report, we were unable to detect pathological changes in IVS1 skeletal muscle cells when using biochemical measurements of lysosomal glycogen content, and immunofluorescent analysis of lysosomal size and number[22]. We hypothesized that lysosomal pathology in skeletal muscle cells from IVS1 patients requires longer periods of time to develop *in vitro*. We anticipated that changes in expression levels of downstream effectors of GAA deficiency might already been affected at an early stage of childhood/adult Pompe disease. Complete restoration of GAA enzyme activity allows filtering for genetic variation in isogenic Pompe disease donors. Here, we also engineered a classic-infantile Pompe disease line where we introduced a healthy copy of the *GAA* cDNA (or an empty copy transgene) in the *AAVS1* safe harbor location as we described previously in a different patient line[18]. Engineered CI myotubes overexpress *GAA* (GAAOE) which resulted in increased enzymatic activity as observed before (figure S2C, D, E). To gain insight into altered expression levels during muscle differentiation, we performed whole transcriptome analyses using genome-wide RNA-sequencing of two LO and two CI isogenic iPSC-derived myotubes. Principal component analysis of all four isogenic groups demonstrated different responses according to disease onset (figure 4A). Both isogenic groups of each disease onset showed similar directionality upon GAA restoration. We generated several expression profiles by filtering differentially expressed genes (DEGs) according to fold change (FC) >1.5 and false discovery rate (FDR) <0.05. First we compared each LO and CI isogenic lines and then we generated and compared a LO dataset vs a CI dataset which resulted in large changes in gene expression, especially in the CI profile, and low number of genes in common between both datasets (figure 4B). Gene set enrichment analysis (GSEA) revealed multiple biological processes differentially enriched for each dataset related to muscle development, glucose and fatty acid metabolism, oxidative phosphorylation and cell division (figure 4C, D).

Representation of all DEGs from both LO and CI datasets in volcano plots illustrate a large variation in gene expression level among isogenic lines (figure 4E, F, S3A). The expression of key genes involved in glycolysis, glycogen synthesis, and the tricarboxylic acid cycle (TCA) were identified among statistically significant DEGs.

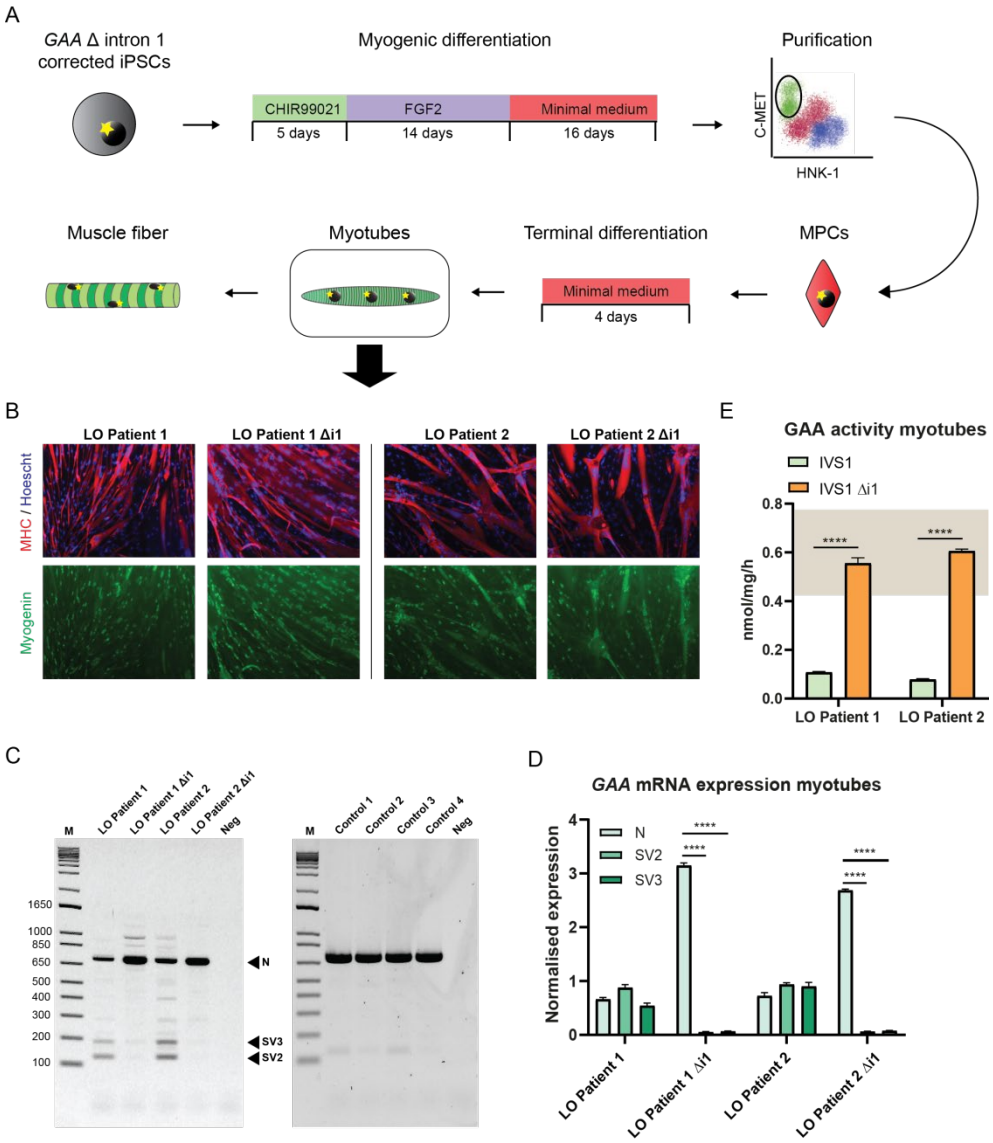


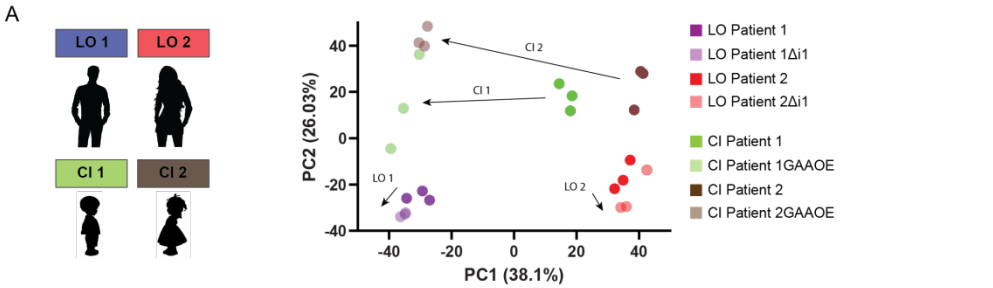
Figure 3. Shortening of GAA intron 1 in iPSC derived skeletal muscle cells results in complete correction of aberrant GAA splicing, GAA mRNA expression and GAA enzyme activity. *(A) Differentiation strategy to generate iPSC derived skeletal muscle cells. Purified myogenic progenitor*

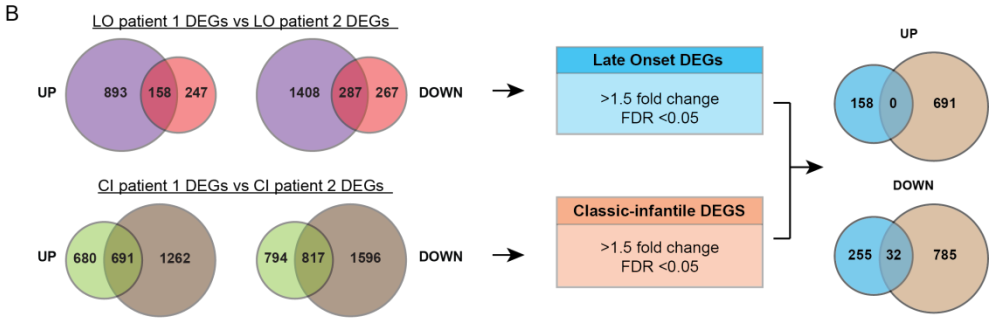
*cells were expanded and differentiated into myotubes as indicated in the experimental setup. (B) Differentiation of MPCs to myotubes from both isogenic LO lines. Myotubes were Stained with α-MHC (red) and α-Myogenin (green) antibodies and nuclei (blue) were visualized with Hoechst. (C) Isogenic LO lines and four healthy control skeletal muscle lines were analyzed with RT-PCR using primers spanning exon 1 – exon 3. Black arrows indicate N, SV3 and SV2 products. (D) Splice product-specific RT-qPCR on the same patient samples as in (C), calculated with the deltaCT method. (E) GAA enzyme activity on isogenic LO myotubes. The colored area indicate the range of GAA activity detected in four different healthy donor-derived myotubes as in figure S2B . Data are means ± standard error of three biological replicates. ns = not significant, * p < 0.05, **p <0.01 and ***p < 0.001.*

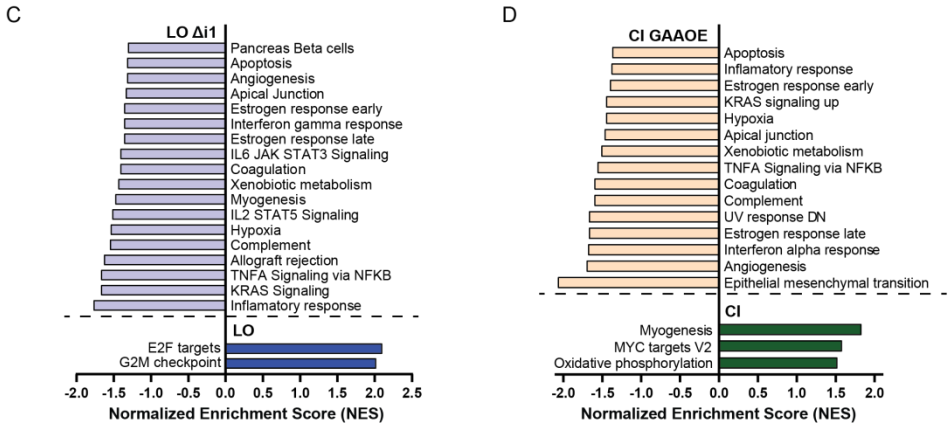
These findings suggested that defects in GAA enzyme activity initiate large transcriptional changes early during muscle differentiation.

Aberrant GAA expression leads to gene expresion changes involved in glucose metabolism, muscle cell contraction and development in differentiating skeletal muscle cells from Pompe patients

In order to get further insight into altered molecular candidates, we next examined the gene expression profile of selected genes implicated in overrepresented biological processes. Heatmaps of genes involved in myogenesis and calcium / ion signaling showed an enrichment of sarcomeric genes, myogenic factors and sarcolemmal ion handling genes in CI Pompe disease myotubes (figure 5A). These results suggest a robust muscle differentiation response upon terminal differentiation, in agreement with several studies showing enhanced muscle regeneration in GAAKO mice after acute injury[31,32]. Additionally, multiple genes involved in glucose metabolism were differentially enriched in both CI patient lines, indicating potential defects in energy production via glucose utilization in absence of GAA activity. Remarkably, the low enrichment of essential genes involved in glucose metabolism in LO Pompe patient lines, strongly points to impaired glucose consumption (figure 5B). Among the genes down-regulated in LO, we could observe enzymes involved in anaerobic glycolysis and glycogen breakdown, as well as glucose and fructose transporters (figure 6A). Notably, anaerobic glycolytic enzymes were not disregulated in CI myotubes (figure 6B). Instead, TCA cycle enzymes were specially enriched, indicating a preference for oxidative phosphorilation in differentiating CI myotubes. Additionally, ezymes involved in cytosolic glycogen synthesis were found up-regulated in CI. Expression of crucial myogenic factors such as *PAX7, MYOD, MYOG* and structural genes involved in


A
LO 1
LO 2
CI 1
CI 2
PC2 (26.03%)
PC1 (38.1%)
LO Patient 1
LO Patient 1Δi1
LO Patient 2
LO Patient 2Δi1
CI Patient 1
CI Patient 1GAAOE
CI Patient 2
CI Patient 2GAAOE


B
LO patient 1 DEGs vs LO patient 2 DEGs
UP
893
158
247
1408
287
267
DOWN
CI patient 1 DEGs vs CI patient 2 DEGs
680
691
1262
794
817
1596
Late Onset DEGs
>1.5 fold change
FDR <0.05
Classic-infantile DEGS
>1.5 fold change
FDR <0.05
158
0
691
255
32
785


C
LO Δi1
Pancreas Beta cells
Apoptosis
Angiogenesis
Apical Junction
Estrogen response early
Interferon gamma response
Estrogen response late
IL6 JAK STAT3 Signaling
Coagulation
Xenobiotic metabolism
Myogenesis
IL2 STAT5 Signaling
Hypoxia
Complement
Allograft rejection
TNFA Signaling via NFKB
KRAS Signaling
Inflamatory response
LO
E2F targets
G2M checkpoint
Normalized Enrichment Score (NES)
D
CI GAAOE
Apoptosis
Inflamatory response
Estrogen response early
KRAS signaling up
Hypoxia
Apical junction
Xenobiotic metabolism
TNFA Signaling via NFKB
Coagulation
Complement
UV response DN
Estrogen response late
Interferon alpha response
Angiogenesis
Epithelial mesenchymal transition
CI
Myogenesis
MYC targets V2
Oxidative phosphorylation
Normalized Enrichment Score (NES)

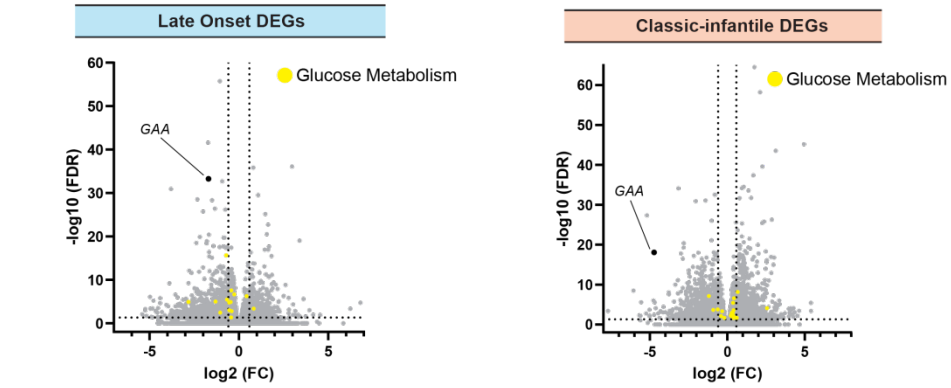

E
Late Onset DEGs
Glucose Metabolism
GAA
-log10 (FDR)
log2 (FC)
F
Classic-infantile DEGs
Glucose Metabolism
GAA
-log10 (FDR)
log2 (FC)

Figure 4. RNA sequencing analysis of differentiating isogenic iPSC-derived skeletal muscle cells from LO and CI Pompe disease reveal large transcriptional changes. *(A) One male and one female lines were evaluated for LO and CI Pompe disease after 4 days of myogenic induction. PCA plot of RNA-seq samples. Colors represent different samples. Arrows indicate isogenic lines. (B)* Venn diagram of up- and down-regulated genes in common for each LO and CI patient lines respectively and in common between LO dataset vs CI dataset. *DEGs were ranked according to FC >1.5 and FDR <0.05. (C) GSEA Hallmark analysis of DEGs from LO dataset and (D) GSEA Hallmark analysis of DEGs from CI dataset ranked according to normalized enrichment score (NES) and highest FDR value. (E) Volcano plots for LO and (F) for CI DEGs. Horizontal dot lines represent FDR =0.05 and vertical lines FC =1.5.Yellow dots represent genes involved in glucose metabolism. Black dots represent the GAA gene.*

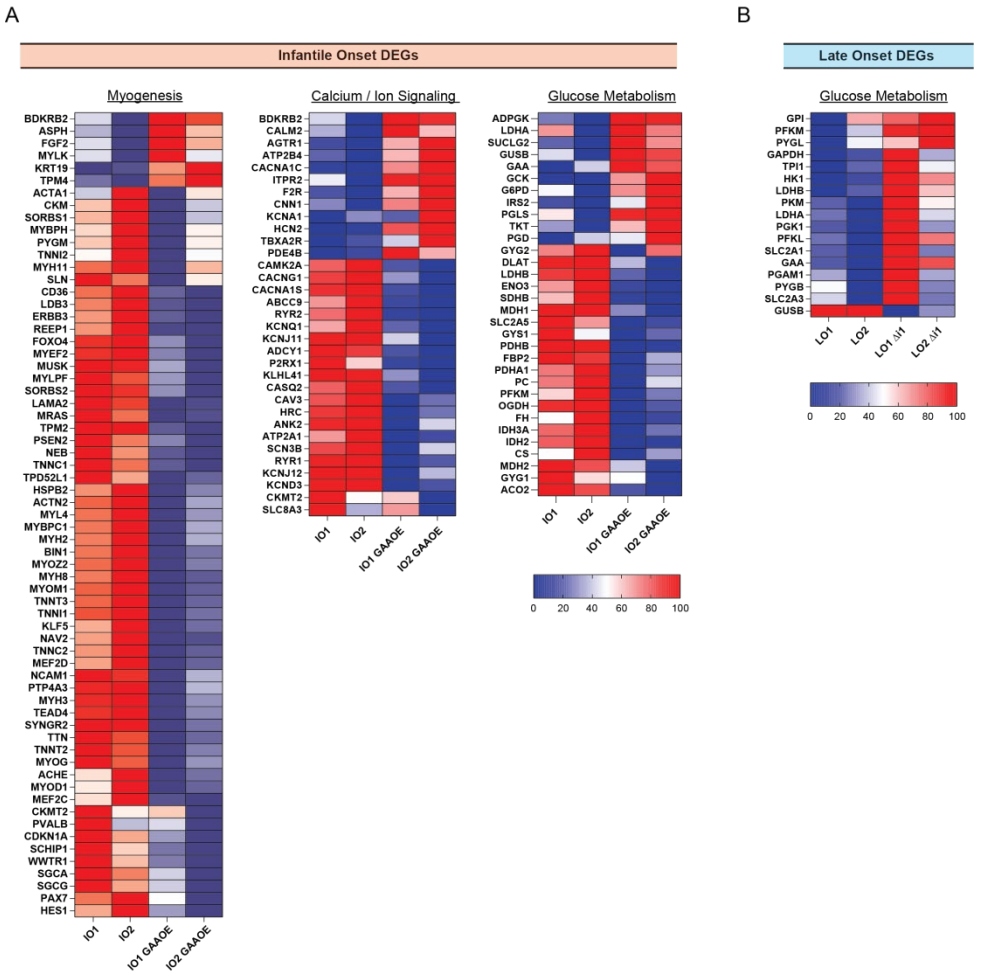


Figure 5. Transcriptional profiling of selected genes indicate altered glucose metabolism, calcium / ion signaling and myogenic progression of differentiating skeletal muscle cells derived from LO and CI Pompe patients. *(A) Heatmaps showing selected gene expression of CI patient and GAAOE lines. (B) Heatmap view of selected gene expression of LO patient and Δi1 lines. Represented genes were statistically significant FDR <0.05.*

contractile force such as *TTN* and multiple *MYH* isoforms was significanlty enhanced in CI myotubes (figure 6C). Taken together, these findings point to major alterations in glucose metabolism routes and skeletal muscle development linked to defects in GAA enzymatic activity in differentiating isogenic Pompe patient muscle cells.

DISCUSSION

The lack of appropriate cellular models of target tissues has hindered our understanding of how *GAA* variants impact disease progression in skeletal muscle cells of Pompe disease patients. In this study, we successfully developed a novel gene-editing intervention to restore GAA enzyme activity levels by shortening the intron 1 of the *GAA* gene in Pompe disease patients carrying the IVS1 variant. We first utilized an IVS1 *GAA* minigene and showed up-regulation of expression and correction of aberrant splicing after removal of 1.9 kb of intron 1, as it was observed before[25]. Raben et al, previously showed enhanced expression of the normal transcript in WT and IVS1 minigenes[25]. However, how shortening of intron 1 affects expression of alternative splicing variants was not studied before. Here, we analyzed alternative splicing in minigenes and detected a different ratio between transcript N, SV2 and SV3, as compared with expression of these transcripts in fibroblasts or skeletal muscle cells with the IVS1 variant[22]. When 1.9 kb were deleted in intron 1, expression of transcript N was more enhanced than SV2 and SV3, indicating correction of aberrant splicing. We next excised 2.1 kb of intron 1 in genomic DNA of two IVS1 patient derived iPSCs using CRISPR/Cas9. The partial removal of intron 1 in LO patient derived iPSCs, revealed an 8.06% of IVS1 allele specific targeting efficiency and a 19.35% in both alleles. Considering that LO Pompe disease patients contain a null variant in the second allele, the summed editing efficiency for the IVS1 allele reached 27,41%, which demonstrates the efficiency of shortening the intron 1 in IVS1 Pompe patients. To better investigate the therapeutic impact of the gene-editing intervention in affected tissues, we generated skeletal muscle cells from patient iPSCs using a directed differentiation method as we described before[18]. Genetically engineered myotubes showed complete restoration of aberrant splicing and enhanced expression of *GAA* result-

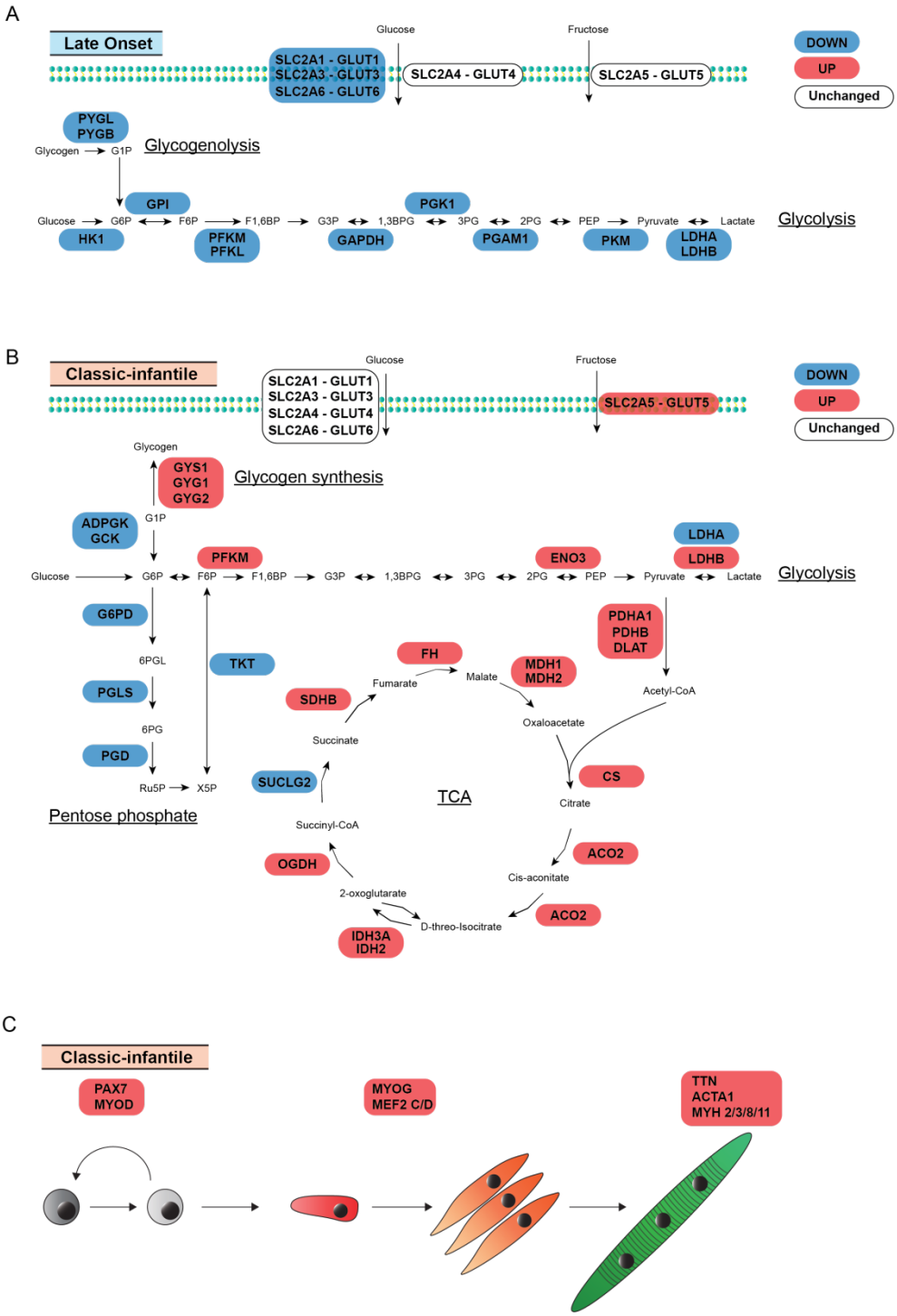

A
Late Onset
Glucose
Fructose
SLC2A1 - GLUT1
SLC2A3 - GLUT3
SLC2A6 - GLUT6
SLC2A4 - GLUT4
SLC2A5 - GLUT5
DOWN
UP
Unchanged
PYGL
PYGB
Glycogen → G1P
Glycogenolysis
GPI
Glucose → G6P ↔ F6P → F1,6BP → G3P ↔ 1,3BPG ↔ 3PG ↔ 2PG ↔ PEP → Pyruvate ↔ Lactate
Glycolysis
HK1
PFKM
PFKL
PGK1
GAPDH
PGAM1
PKM
LDHA
LDHB
B
Classic-infantile
Glucose
Fructose
SLC2A1 - GLUT1
SLC2A3 - GLUT3
SLC2A4 - GLUT4
SLC2A6 - GLUT6
SLC2A5 - GLUT5
DOWN
UP
Unchanged
Glycogen
GYS1
GYG1
GYG2
Glycogen synthesis
G1P
ADPGK
GCK
LDHA
LDHB
PFKM
ENO3
Glucose → G6P ↔ F6P → F1,6BP → G3P ↔ 1,3BPG ↔ 3PG ↔ 2PG ↔ PEP → Pyruvate ↔ Lactate
Glycolysis
G6PD
6PGL
PGLS
6PG
PGD
Ru5P → X5P
Pentose phosphate
TKT
PDHA1
PDHB
DLAT
FH
MDH1
MDH2
Fumarate
Malate
SDHB
Acetyl-CoA
Succinate
Oxaloacetate
CS
SUCLG2
TCA
Citrate
Succinyl-CoA
ACO2
OGDH
Cis-aconitate
2-oxoglutarate
ACO2
D-threo-Isocitrate
IDH3A
IDH2
C
Classic-infantile
PAX7
MYOD
MYOG
MEF2 C/D
TTN
ACTA1
MYH 2/3/8/11

Figure 6. Schematic models of metabolic routes indicating distinct glucose utilization and muscle differentiation capacities in late onset and classic-infantile Pompe disease myotubes. *(A) Altered glucose metabolism genes in late onset. (B) Altered glucose metabolism genes in classic-infantile. Blue indicate down-regulated genes, red up-regulated genes and white unchanged. (C) Selected up-regulated genes involved in myogenesis in classic-infantile myotubes. Represented genes were statistically significant FDR <0.05.*

-ing in a GAA enzyme activity comparable to two wild type alleles. To our knowledge, we are the first to remove part of an intron, leaving both the canonical splice sites and the pathogenic variant in the engineered allele intact, and demonstrate enhanced targeted gene expression. Currently, the number of regulators identified for GAA is limited, making it difficult to predict potential interactions that can be associated with the skeletal muscle wasting phenotype. To further study this, protein expression analysis should be performed on gene-corrected skeletal muscle cells, and interactions between GAA and potential regulators should be determined. The transcription factor TFEB is involved in the regulation of several genes from the lysosomal compartment, including GAA[33]. HES1 and YY1 transcription factors, are so far the only known regulators for the *GAA* gene[26,27,34]. YY1 plays a critical role in skeletal muscle regeneration and embryonic muscle formation[35]. Loss of YY1 has been linked to complete abrogation of satellite cell activation upon damage, which naturally represses mitochondrial gene expression in favor of glycolysis. Similarly, HES1 plays a role in muscle development and regeneration by regulating the expression of MYOD in proliferating satellite cells[36]. Here, we only found HES1 gene enrichment in CI myotubes. It can be hypothesized that a feedback mechanism regulates *GAA* expression in cells, and that low GAA enzyme activity results in altered binding of HES1 and/or YY1 factors at the intron 1 of *GAA*.

Late onset disorders are characterized by slow progression of pathological alterations and heterogeneous severity of disease symptoms. These can make it difficult to identify and study of disease-related mechanisms *in vitro*. The generation of isogenic models could greatly diminish the interference created by (epi)genetic variation among individuals. These methods have provided valuable insight into brain disorders[37,38], retinal degeneration[39] and heart diseases[40–44]. The

GAA enzyme is needed for glycogen breakdown in the lysosomes. Lysosomal accumulation of glycogen occurs in individuals with Pompe disease. The severity of the disease varies based on the type of *GAA* variants and deficiency of GAA. Detection of pathological hallmarks such as glycogen accumulation is in particular difficult in cells of late onset Pompe disease patients due to the slow progression of the disease pathology. Here, we have exploited two different gene-editing strategies to restore GAA enzyme activity in two late onset (Δ intron 1) and two classic-infantile Pompe patient iPSCs (GAA overexpression) and investigated genome-wide expression changes during skeletal muscle differentiation in patient derived cells *in vitro*. Our study revealed that despite the lack of robust disease pathology in cells, large gene expression changes occurred early during myogenic differentiation. GSEA transcriptome analysis indicated altered metabolic and muscle differentiation processes in both LO and CI Pompe disease lines. Changes in myogenesis were most evident in myotubes derived from classic-infantile Pompe patients. Our transcriptome profiling experiments indicated an enhanced muscle differentiation response in CI disease lines in contrast to the isogenic gene-corrected ones. This finding is in accordance with recent literature describing a rapid regenerative response in the skeletal muscles of GAAKO mice after acute muscle injury[31,32]. Similarly, crucial transcription factors involved in myogenesis such as PAX7, MYOD, MYOG and MEF2 where enriched in differentiating CI muscle cells. These results point to a rapid progression through the myogenic lineage. However, this initial increase in satellite cell number was not identified in classic-infantile and childhood/adult onset muscle biopsies of Pompe disease patients under homeostatic conditions[45]. While satellite cell expression seems unaltered in patient biopsies, enhanced Pax7 expression was identified in regenerating muscles of GAAKO mice when compared to WT[32]. Further studies could elucidate whether the physiological response of satellite cells vary according to the disease stage under muscle regeneration in long-cultured *in vitro* models. In addition, multiple genes from the sarcomere were robustly enriched in CI myotubes. Sarcomeric genes such as TNNT2, MYH3 and MYH8 have been similarly identified in transcriptomic studies performed in muscle biopsies of classic-infantile Pompe patients compared to healthy controls[46]. Our study

additionally revealed other sarcomeric genes differentially expressed such as TTN, ACTA1, MYH2/11, TNNC1/2, MYBPC1, TNNT3 and TPM1/4 among others with known implications in skeletal muscle function. Several studies have previously reported aberrant calcium influx in GAAKO mouse myotubes, indicating that calcium accumulation might precede mitochondrial defects and autophagic buildup in classic-infantile Pompe patients[47–49]. Here, we have also identified numerous genes related to calcium but also to sodium and potassium transport, suggesting much profound alterations in contractile force transmission. These findings point to a robust myogenic response in differentiating CI skeletal muscle cells, not found in LO myotubes, and indicate differences in muscle development according to disease onset in Pompe disease. These could be related to GAA enzymatic activity levels and glycogen accumulation previously identified in CI but not in LO myotubes.

Aberrant glucose metabolism has been extensively studied using KO mouse models of Pompe disease[48,50–54]. Despite the existence of conserved genes and physiological responses between rodents and humans, significant species-specific differences might limit the translational potential of drug candidates for clinical use[55,56]. Only few studies have recently used patient-derived cells to study defects of altered metabolism in late onset Pompe patients[57,58]. However, cardiac abnormalities are rare in late onset Pompe patients and therefore cardiomyocytes do not represent the most suitable cell type[59]. Meinke et al, demonstrated a functional reduction in glycolysis in late onset patient-derived myoblasts when compared to ERT treated and control lines[58]. However, the variability derived from using multiple patient and control lines increase the heterogeneity of results achieved. Here, using two different isogenic control lines, we identified a significant down-regulation of glycolytic enzymes in LO myotubes. No mitochondrial respiration related genes were substantially deregulated in the LO profile, which could be due to the embryonic/fetal genetic signature of cells derived from iPSCs[18,60]. These findings indicate that glycolytic defects are the first abnormalities present in differentiating LO muscle cells which could precede others typically found in skeletal muscles of late onset Pompe patients such as energy defects and autophagic buildup. Surprisingly, we could not find a profound impact in glycolytic

enzymes in differentiating CI muscle cells as others have previously found in mice[51,53,54], except for a down-regulation of pentose phosphate enzymes. However, we could observe an enrichment of glycogen branching enzymes, similarly observed in mice studies before[50,51,54]. These results suggest an up-regulation of glycogen synthesis in CI patient myotubes, hence, strategies directed to reduce cytosolic glycogen could be of therapeutic benefit for classic-infantile patients. Another interesting aspect that our findings revealed is the up-regulation of genes involved in the TCA cycle of CI myotubes. We could hypothesize that the enrichment in mitochondrial respiration could be coupled to their rapid myogenic progression. A recent study from Meena et al, documented higher levels of TCA related metabolites in skeletal muscles of GAAKO mice[54]. However, conflicting results have been presented regarding mitochondrial respiration defects in Pompe mice among several studies[48,57,58,61]. The heterogeneity of results obtained could be explained by the different lines and culture methods used in these studies.

Taken together, we demonstrated that shortening the intron 1 of the *GAA* gene restored aberrant splicing and enzymatic activity levels in IVS1 LO Pompe disease skeletal muscle cells. Here, we further proved the capacity of tailored isogenic controls to identify early pathological changes in differentiating skeletal muscle cells from LO and CI Pompe patients. With the advent of innovative 3 dimensional culture platforms, future assays will allow determining functional properties in more physiologically mature skeletal muscle models derived from patient iPSCs[62]. This research revealed changes in glucose metabolism and muscle development linked to GAA using iPSC-derived skeletal muscle cells from Pompe patients.

METHODS

Construction of the GAA exon 1-3 minigene

Genomic *GAA* DNA region of *GAA* exon 1–3 (chr17:80101704-80105894, GRCh38.p7) from a healthy control was amplified with Phusion® High-Fidelity PCR Kit (Thermo Scientific, Waltham, MA) and cloned into pcDNA3.1(-)Myc-His A vector using XbaI and NotI restriction sites. 1.9 kb of intron 1 was removed by using BsmBI and SbfI restriction sites, followed by a klenow reaction and a ligation step.

The IVS1 variant (c.-32-13T>G) was introduced using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies). Final constructs were validated with restriction enzyme analysis and sequencing.

Culture of HEK293T cells

HEK293T cells were cultured in DMEM High glucose (Gibco, Waltham, MA) supplemented with 10% Fetal Bovine Serum (Thermo Scientific, Waltham, MA) and 100 U/ml penicillin streptomycin (Gibco, Waltham, MA). HEK293T cells were plated with 40.000 cells in 12 wells and 24 hours later transfected with 1 µg plasmid using Fugene 6 (Promega, Fitchburg, WI) transfection reagent according to manufacturer's manual. RNA was harvested after 72 hours of transfection.

Culture and differentiation of iPSCs into MPCs

Patient lines used for this study include the following genotypes: LO1 c.-32-13T>G/525del (XY); LO2 c.-32-13T>G/525del (XX); CI1 c.525del/525del (XY); CI2 c.2481+102_2646+31del (XX). Reprogramming and feeder-based culture of iPSCs was performed according to Warlich et al,[28]. iPSCs were differentiated into myogenic progenitor cells (MPCs) and expanded as described in van der Wal et al,[18,22]. Briefly, differentiation of iPSCs into myogenic progenitor cells (MPCs) was started on 10 cm dish with a 40% confluent culture, grown for 5 days in iPSC medium followed by the myogenic differentiation procedure consisting of: 5 days myogenic differentiation medium (DMEM/F12, 1% Insulin-Transferrin-Selenium-Ethanolamine, 1% penicillin/streptomycin/L-glutamine, all Gibco, Waltham, MA) supplemented with 3.5 µM CHIR99021 (Axon Medchem, Groningen, The Netherlands), 14 days myogenic differentiation medium supplemented with 20 ng/ml FGF2 (Prepotech, Rocky Hill, NJ) and 16 days myogenic differentiation medium only. For purification, MPCs were sorted with FACS with anti-C-MET-APC (1:50, R&D systems, Minneapolis MN) and anti-HNK-1-FITC (1:100, Aviv Systems Biology, San Diego, CA) antibodies. Purified MPCs were expanded in MPCs proliferation medium consisting of DMEM high glucose supplemented with 10% Fetal Bovine Serum, 100 U/ml penicillin/streptomycin and 100 ng/ml FGF2. Differentiation of MPCs into skeletal muscle cells was started when MPCs reached

a confluency of 90%, by switching to myogenic differentiation medium. After 4 days skeletal muscle cells were harvested.

Gene editing of iPSCs

Single guide RNA (sgRNA) sequences were designed using CRISPRscan program to select optimal target sites for intron 1 (table S1). Selected sgRNA sequences were inserted in a TOPO vector containing the U6 promoter (addgene: 41824). Prior gene editing of iPSCs, all sgRNAs were first tested in HEK293T cells. Confluent iPSCs on feeders were pretreated 4 hours before nucleofection with 10 μM Rock inhibitor (Y-27632 dihydrochloride, Ascent Scientific, Asc-129). Single cells were generated from iPSC colonies by incubating with Accutase (Thermo Scientific, Waltham, MA), and $2*10^6$ cells were nucleofected with 3.6 μg pCAG-hCAS9-GFP (addgene: 44719) and 1.8 μg of each TOPO-sgRNA using Amaxa Human Stem Cell Nucleofector Kit2 (VPH-5022) with program B-016. After nucleofection, cells were recovered in iPSC-conditioned medium (iPSC medium incubated for 24 hours on feeder cells) supplemented with 20 ng/ml FGF2 and 10 μM rock inhibitor. After 48 hours, single iPSCs were plated in a dilution range to obtain single iPSC colonies.

Genotyping of single iPSC colonies

Approximately, 14 days after plating single iPSCs, single colonies were picked and expanded in 2x 48 wells containing feeders. After 7 days, 1 well was sacrificed for genotyping. Genomic DNA was extracted using a high salt method. Positive iPSC clones were expanded and tested. PCR and sequencing was used to determine the purity of iPSC clones, and to identify the targeted allele and the position of the cleavage site. The PCR reaction consisted of 12.5 ng genomic DNA with 0.4 μL Faststart Taq Polymerase (Roche, Penzberg, Germany), 0.33 mM dNTPs, 0.33 μM forward and reverse primers in a 15 μL reaction (table S2).

RNA isolation and cDNA synthesis

RNA was isolated using the RNeasy minikit with DNAse treatment (Qiagen, Germantown, MD). Isolated RNA was stored at -80 °C. 500 ng of RNA was

synthesized into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to manufacturer's protocol.

qRT-PCR

qRT-PCR was measured with a CFX96 real-time system (Bio-Rad, Hercules, CA). cDNA was diluted 5x or 10x times and 4 µL was used in a qRT-PCR reaction consisting of a total volume of 15 µL with 7.5 µL iTaq Universersal SYBR Green Supermix (Bio-Rad, Hercules, CA), 10 pmol/µl forward and reverse primers (Table S2). For each plate a standard curve was included with 5 dilutions. Gene expression was normalized using each of the following four genes: MyoD, Myogenin, Lamp1 and Lamp2. Thereafter, the average value of the four normalized expression values was calculated.

Flanking exon RT-PCR

Flanking RT-PCR was performed with 2 µL of 10x diluted cDNA with 10 pmol/µl forward and reverse primers (table S2) using the Advantage GC 2 PCR kit (Clontech, Kusatsu, Japan) with a GC-melt concentration of 0.5 M according to the manufacturer's instructions. A 1.5% agarose gel containing 0.5 µg/ml ethidium bromide (Sigma Aldrich, Irvine, UK) was used to analyze the whole RT-PCR reaction.

GAA Enzyme activity assay

Skeletal muscle cells were harvested with ice cold lysis buffer (50 mM Tris (pH 7.5), 100 mM NaCl, 50 mM NaF, 1% Triton X-100 and one tablet Protease Inhibitor Cocktail cOmplete, with EDTA, (Roche, Penzberg, Germany)) for 10 minutes on ice. GAA enzyme activity was measured using 4-methylumbelliferyl α-D-glucopyranoside substrate (Sigma Aldrich, Irvine, UK) as described previously[8]. Total protein concentrations was determined with the BCA protein assay kit (Pierce, Thermo Scientific, Waltham, MA).

RNA sequencing and transcriptome analysis

RNA patient samples were sequenced in triplicates according to van der Wal et al,[18]. Analysis was performed with new Tuxedo pipeline[63]. Briefly, FASTQ RNA

sequencing files were aligned to HG38 (igenome, Illumina, San Diego, CA) using HISAT2 and assembled with Stringtie. FPKMs were calculated with the ballgown package. Then, differentially expressed genes were ranked according to fold change >1.5 and FDR <0.05 based on the comparison of each individual isogenic pair and depicted in Venn diagrams and volcano plots. GSEA is a publicly available software to statistically evaluate genome wide expression differences based on Gene Ontology (GO) and KEGG pathways among other gene set databases. For this analysis, we used the Hallmark collection, consisting of curated gene sets derived from multiple GO and KEGG sets as described in[64]. We performed the analysis using the following settings: 1000 permutations, gene-set permutation type, weighted enrichment statistics and signal2noise as metric for ranking genes. We evaluated the biological relevance of the input genes based on normalized enrichment score (NES) and FDR.

Immunofluorescence

Cultured skeletal muscle cells were fixed with 4% paraformaldehyde (Merck, Kenilworth, NJ) in PBS for 10 minutes at room temperature (RT) and stored in PBS at 4 °C. For immunofluorescence cells were permeabilized for 5 minutes with 0.1% Triton X-100 (AppliChem, Darmstadt, Germany) in PBS and blocked for 30 minutes at RT with blocking solution (PBS, with 0.1% Tween and 3% BSA, all Sigma Aldrich, Irvine, UK). Rabbit-α-Myogenin (Santa Cruz, sc-576, 1:100) and Mouse-α-MF20 (DSHB, 1:50) were diluted into 0.1% BSA in PBS-T (PBS with 0.1% Tween) and incubated for 1 hour at RT. After incubation cells were washed (1x PBS-T and 1x PBS both for 2 minutes) and incubated with the secondary antibodies (Alexa-Fluor-488-α-rabbit 1:500, Invitrogen, Carlsbad, CA and horse anti-mouse biotin, Vector Laboratories, Burlingame, CA) in PBS-T for 30 minutes at RT. For incubations with secondary biotinylated antibodies cells were washed and incubated with Streptavidine 594 (1:500, Invitrogen, Carlsbad, CA,). The cells were washed and incubated for 15 minutes with Hoechst (1:15000, Thermo Scientific, Waltham, MA) and imaged in PBS.

Statistical analysis

Data were analyzed and represented using Prism 8 (GraphPad). Statistical assessment was performed using ANOVA test. Data are means ± standard deviation of three biological replicates. * $p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

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SUPPLEMENTARY FIGURES AND TABLES

Figure S1. ***Neomycin*** **expression of transfected minigenes.** Expression of neomycin was quantified using qRT-PCR after transfection of HEK293T cells with WT, WT ∆i1, IVS1 and IVS1 ∆i1 minigenes. One way Anova detected no significant change in expression between the samples.

Figure S2. **GAA expression and activity in patient myotubes.** (A) Slow cool down of potentially folded RT-PCR products. Red arrow indicate shifted band. (B) GAA activity measured in 4 different healthy-derived myotubes. (C) *GAA* expression in classic-infantile patient and gene-corrected myotubes. (D) GAA enzyme activity in classic-infantile patient and gene-corrected myotubes. (E) Immunofluorescence images of myotubes stained with α-MHC (red) and α-Myogenin (green) antibodies and nuclei (blue) visualized with Hoechst.

Figure S3. **DEGs from each late onset and classic-infantile patient lines.** (A) Volcano plots of DEGs enriched in LO patient 1 and 2, and CI patient 1 and 2. DEGs were ranked according to FC >1.5 and FDR <0.05. Horizontal dot lines represent FDR =0.05 and vertical lines FC =1.5. Black dots represent the GAA gene.

Figure S1

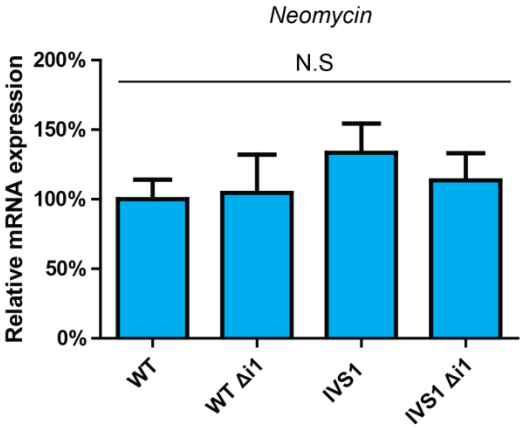
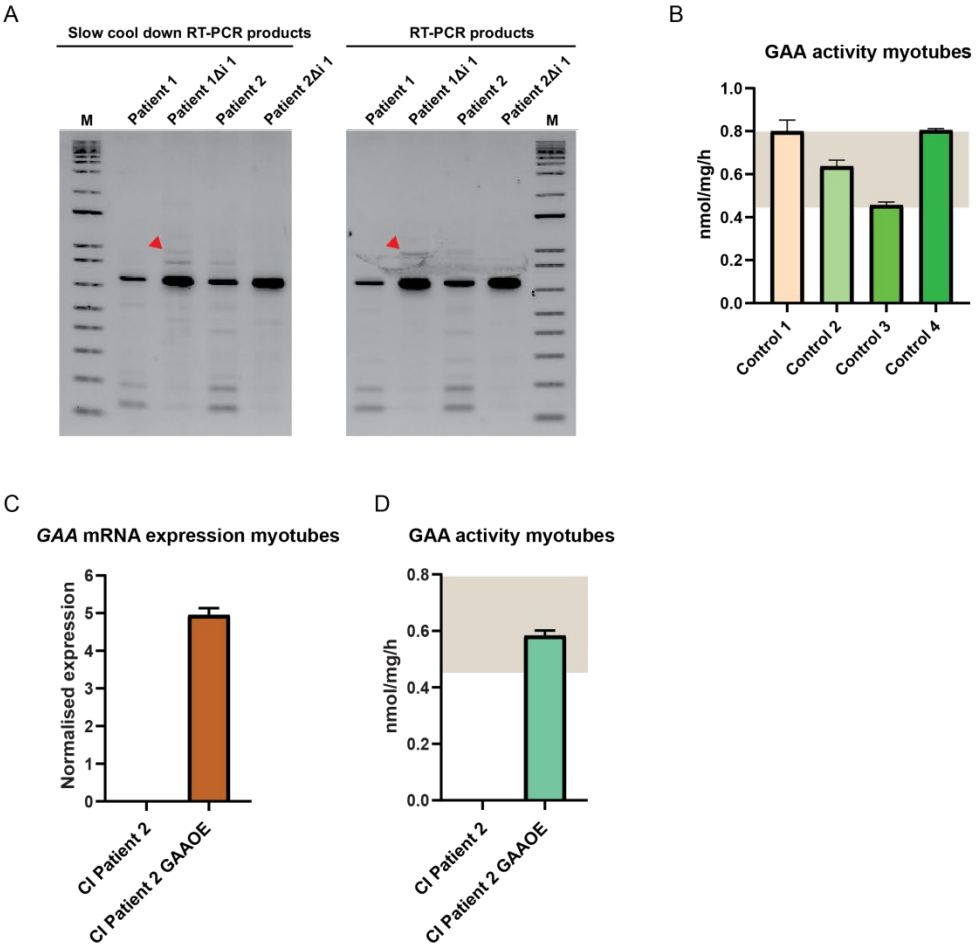


Figure S2


A
Slow cool down RT-PCR products
RT-PCR products
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Patient 1
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B
GAA activity myotubes
nmol/mg/h
1.0
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Control 1
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Control 3
Control 4
C
GAA mRNA expression myotubes
Normalised expression
6
5
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GAA activity myotubes
nmol/mg/h
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MHC / Hoescht
Myogenin

Figure S3

A

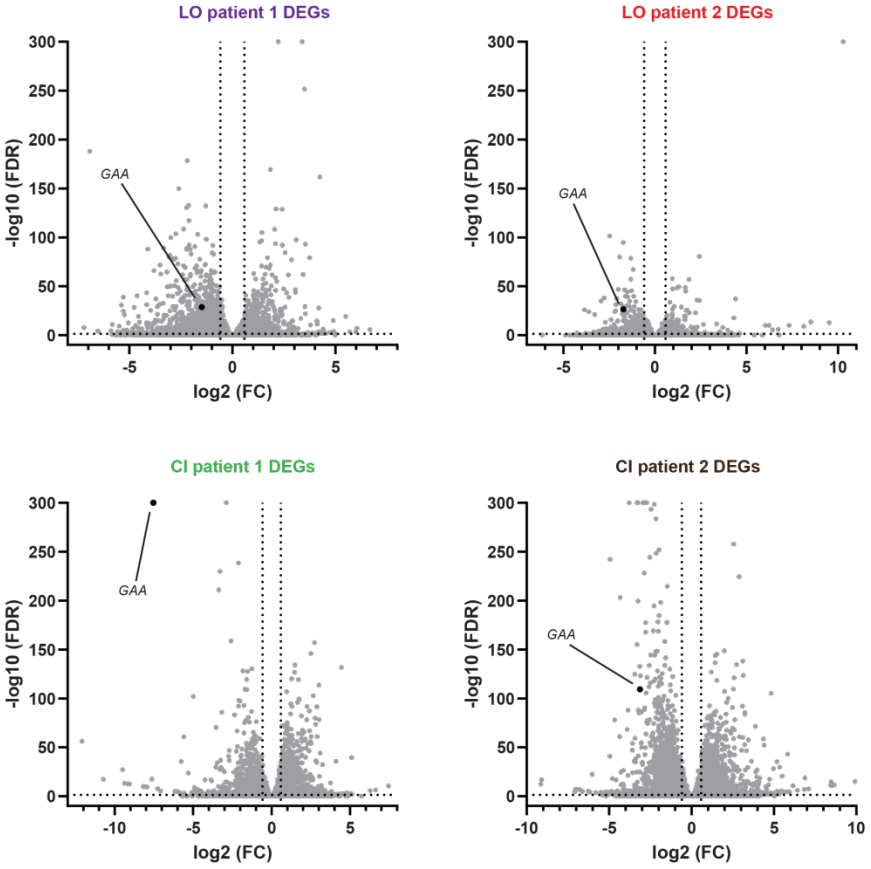

Table S1. **Single guide RNAs used to remove 2.1kb of intron 1**

Position	**Sequence ‘5-‘3**
Left	CCGTGGCCTGAGAGGGGGCCCC
Right	CCCTGCTGGAGCTTTTCTCGC

Table S2. **Primers used for RT-qPCR, RT-PCR and PCR**

Name	**Sequence ‘5-‘3**	**Assay**
GAA Exon 1-2 fw	AAACTGAGGCACGGAGCG	RT-qPCR
GAA Exon 1-2 rv	GAGTGCAGCGGTTGCCAA	RT-qPCR
GAA Cryptic Exon 2 fw	GGCACGGAGCGGGACA	RT-qPCR
GAA Cryptic Exon 2 rv	CTGTTAGCTGGATCTTTGATCGTG	RT-qPCR
GAA Full Skip Exon 2 fw	AGGCACGGAGCGGATCA	RT-qPCR

GAA Full Skip Exon 2 rv	TCGGAGAACTCCACGCTGTA	RT-qPCR
MyoD fw	CACTCCGGTCCCAAATGTAG	RT-qPCR
MyoD rv	TTCCCTGTAGCACCACACAC	RT-qPCR
Myogenin fw	CACTCCCTCACCTCCATCGT	RT-qPCR
Myogenin rv	CATCTGGGAAGGCCACAGA	RT-qPCR
Lamp 1 fw	GTGTTAGTGGCACCCAGGTC	RT-qPCR
Lamp 1 rv	GGAAGGCCTGTCTTGTTCAC	RT-qPCR
Lamp 2 fw	CCTGGATTGCGAATTTTACC	RT-qPCR
Lamp 2 rv	ATGGAATTCTGATGGCCAAA	RT-qPCR
GAA Exon 1a_GC fw	CGAGCTCCCGCCGGTCACGTGACCC	PCR 1, determines editing outcome. Also for IVS1 specific PCR 2. Fig 2C.
GAA Exon 3_GC rv	TCCAAGGGCACCTCGTAGCGCCTGTTA	PCR 1, determines editing outcome. Fig 2C.
IVS1 allele_GC rv	GCTCCTACAGGCCTGCGGGAGAAGC	PCR 2, IVS1 specific. Fig 2C.
Fw_in1_gDNA	TGGGAAAGCTGAGGTTGTCG	PCR, initial screening. Fig 2B
Rv_in1_gDNA	CAGCTCTGAGACATCAACCG	PCR, initial screening. Fig 2B.
Fw_primer_1	CATGGCTGGGTCTGAATCCC	PCR, specific for Δ intron 1 product. Fig 2D.
Fw_primer_2	TACCTGCCTTGCTGGTCTTC	PCR, specific for WT product. Fig 2D.
Rv_primer_3	GGTGAGTCTCCTCCAGGACT	PCR. Fig 2D.
Fw_delta_intron1_bp_seq	CAGAAGCGGGTTTGAACGTG	PCR, determines cleavage site. Fig 2F.
Rv_delta_intron1_bp_seq	GGAGAAGAAAGCGGGCTCAG	PCR, determines cleavage site. Fig 2F.
GAA_Exon1b_GC fw	AGGTTCTCCTCGTCCGCCCGTTGTTCA	RT-PCR
GAA_Exon3_GC rv	TCCAAGGGCACCTCGTAGCGCCTGTTA	RT-PCR

Chapter 7

Engineered human skeletal muscles to study injury and regeneration *in vitro*

Engineered human skeletal muscles to study injury and regeneration *in vitro*

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In preparation

ABSTRACT

Satellite cells become rapidly activated to efficiently regenerate skeletal muscle tissues after injury. Muscle regeneration has been extensively studied in animal models *in vivo*. However, how this process occurs in humans is not well understood. Current models are hampered by inappropriate cell and study systems to study functional physiological responses of human skeletal muscle tissues *in vitro*. Here, we sought to overcome this limitation by investigating muscle regeneration using physiologically relevant models of skeletal muscle derived from human iPSCs. Engineered muscles injured by cryodamage resulted in severe muscle wasting while chemically and myotoxin injured muscles showed a less severe damage. Myotoxin injured muscles resulted in robust dose dependent degenerative response suitable to study tissue regeneration. Engineered muscles made of purified muscle progenitor cells contained Pax7+ satellite cells that failed to respond to all myotoxin induced injury *in vitro*. This was in agreement with a requirement of a transient inflammatory response for muscle regeneration, which was lacking in our system. We conclude that the engineered muscle system employed here based on purified muscle cells provides a controlled model system for studying the signals required for the regeneration of human skeletal muscle. Engineered muscle models might ultimately be used to develop drugs that could enhance muscle regeneration in health and disease and increase our understanding of tissue repair.

Key words

Human iPSCs, engineered skeletal muscle, muscle regeneration

INTRODUCTION

Skeletal muscle has a remarkable capacity to efficiently regenerate when damage occurs[1]. The regenerative capacity of the skeletal muscle resides in tissue-specific stem cells known as “satellite cells” (SCs)[2]. In homeostatic conditions, SCs remain quiescent under the basal lamina of muscle fibers and express paired box 7 (Pax7)[3]. When SCs become activated (e.g. due to muscle injury), they rapidly re-enter the cell cycle and asymmetrically divide to generate a large proliferative progeny which eventually fuse with each other and with existing fibers to regenerate muscle. Concomitant to SC differentiation, a fraction of diving SCs retain Pax7 expression to maintain the stem cell pool. However, this robust regenerative response can be impaired under pathological conditions such as volumetric muscle loss and muscle degenerative diseases.

Skeletal muscle regeneration has been predominantly studied using animal models (mostly rodents). The extensive use of animal models has greatly increased our understanding of skeletal muscle biology in health and disease. However, they have shown limited capacity to predict efficacy and safety of drug candidates to treat skeletal muscle disorders. Recent developments based on tissue engineering approaches have allowed researchers to investigate complex human disorders in physiologically relevant models[4] . Different strategies are currently used to recreate the physiological properties of skeletal muscle tissues *in vitro*[5]. Initially, muscle cells from animal origin were used to develop *in vitro* tissue-engineered skeletal muscles (TESMs)[6–9]. Only recently, engineered skeletal muscles have been successfully generated using muscle cells from human origin such as primary cells or iPSC-derived[10–16]. Primary muscle cell models are however scarce, difficult to obtain, and with limited expansion potential. Additionally, muscle cells isolated from biopsies are often contaminated with other non-myogenic cells (neuronal, immune, endothelial, fibroblast) which can influence the experimental results obtained from tissue-engineered muscles. Human iPSCs instead, can be obtained from less invasive material (e.g. skin, blood, urine), have unlimited expansion potential, can be efficiently genetically-corrected and can be differentiated into pure, expandable skeletal muscle cells[17].

Here, we have used tissue-engineering to successfully recreate three-dimensional (3D) skeletal muscles using human iPSC-derived muscle cells and study tissue response to injury. Skeletal muscle tissues can respond differently to injury depending on the method used[18]. To this end, we investigated three different injury methods: cryoinjury (freeze-thaw), chemical injury (barium chloride) and myotoxin injury (cardiotoxin). These have been studied in mice as pre-conditioning regimes to increase muscle cell engraftment after transplantation[19]. Some of these, such as cryoinjury, could be used in the future for regenerative medicine. We first applied cryoinjury to TESMs, which is known to severely damage the muscle architecture and its multiple cellular components. Cryoinjured TESMs similarly demonstrated muscle fiber degradation and SC depletion locally in the damaged area. Other types of injury which do not rely on mechanical stress involve chemicals and myotoxins. Injured TESMs using chemicals such as barium chloride ($BaCl_2$) could replicate muscle fiber degradation although it has low solubility at high concentrations. Notably, precipitated $BaCl_2$ crystals could impair any regenerative response when not efficiently removed from engineered muscles after injury. Myotoxins such as cardiotoxin (CTX) were easily dissolved and exerted a robust dose-dependent damage in injured TESMs. While cryoinjured TESMs resulted in a major loss of cells expressing Pax7, CTX-injured muscles instead led to an intact pool of Pax7 positive cells that could initiate a regenerative response. Efficient muscle regeneration is successfully achieved in coordination with multiple non-myogenic cell types that regulate SC proliferation and differentiation[20]. As expected, TESMs were unable to regenerate after CTX injury. This is likely caused by the lack of cytokines and immune cells that normally induce the muscle regenerative response in the TESM system employed here. As opposed to engineered muscle generated from primary biopsies, which contains multiple cell types, our TESM system made of purified muscle cells may provide a 'clean' system for the future study of immune signals involved in muscle regeneration. Together, these results provide evidence for a suitable platform to study human skeletal muscle injury *in vitro* which could be used in the future to increase our understanding of tissue repair and to develop a muscle regenerative therapy.

RESULTS

Severe muscle damaged induced by freeze-thaw injury conducted in TESMs in vitro

We have previously developed a method for the generation of 3D printed platforms of multiple sizes to generate human iPSC-derived skeletal muscles[10]. Engineered muscles were made of skeletal muscle cells derived from a healthy donor using a previously published directed differentiation protocol for the generation of pure, expandable muscle cells from iPSCs[17]. To replicate the native architecture and promote adequate contractile functions, 3D platforms were made of flexible pillars using polydimethylsiloxane (PDMS) which fit in 24 well plates (figure 1A). First, muscle cells were embedded in a mixture of matrigel and fibrin and cultured in a rocker. TESMs were generated from proliferative muscle cells that were differentiated in the 3D platforms for 7 days. At this time point, TESMs presented aligned multinucleated TITIN+ muscle fibers and a large pool of PAX7+ cells (figure 1B). Cryoinjury lesions are characterized by acute and diffusive cellular necrosis commonly designated as “dead zone”. These are typically conducted using tools that can maintain extremely low temperatures (e.g. copper or steel probes) which are chilled in liquid nitrogen and placed in direct contact with exposed muscles[21]. The severity of the dead zone depends on the period of exposure to the cryoprobe. Injured muscles via freeze damage have the advantage that they allow the study of cell infiltration and regeneration from the non-damaged area. In order to achieve a similar muscle damage as observed in previous studies *in vivo*, we decided to use tools whose size did not exceed the size of the engineered muscles (TESMs average width 17µm, average length 3.25mm). TESMs were cryoinjured and left to recover for one day. Large cryoprobes (3mm) resulted in a wide necrotic area depleted of TITIN+ fibers and PAX7+ cells as expected (figure 1B). However, regeneration cannot occur if no PAX7+ muscle stem cells are present in the muscle[22]. We then used a fine cryoprobe (0.8mm) in order to achieve local muscle damage. We could observe how the necrotic area was restricted to the region in contact with the fine tool, while the non-damaged area resulted in intact muscle fibers and muscle stem cells (figure 1B). These results demonstrated the feasibility

of injury methods like cryodamage to generate local muscle damage to investigate cell infiltration and muscle regeneration after acute injury *in vitro*.

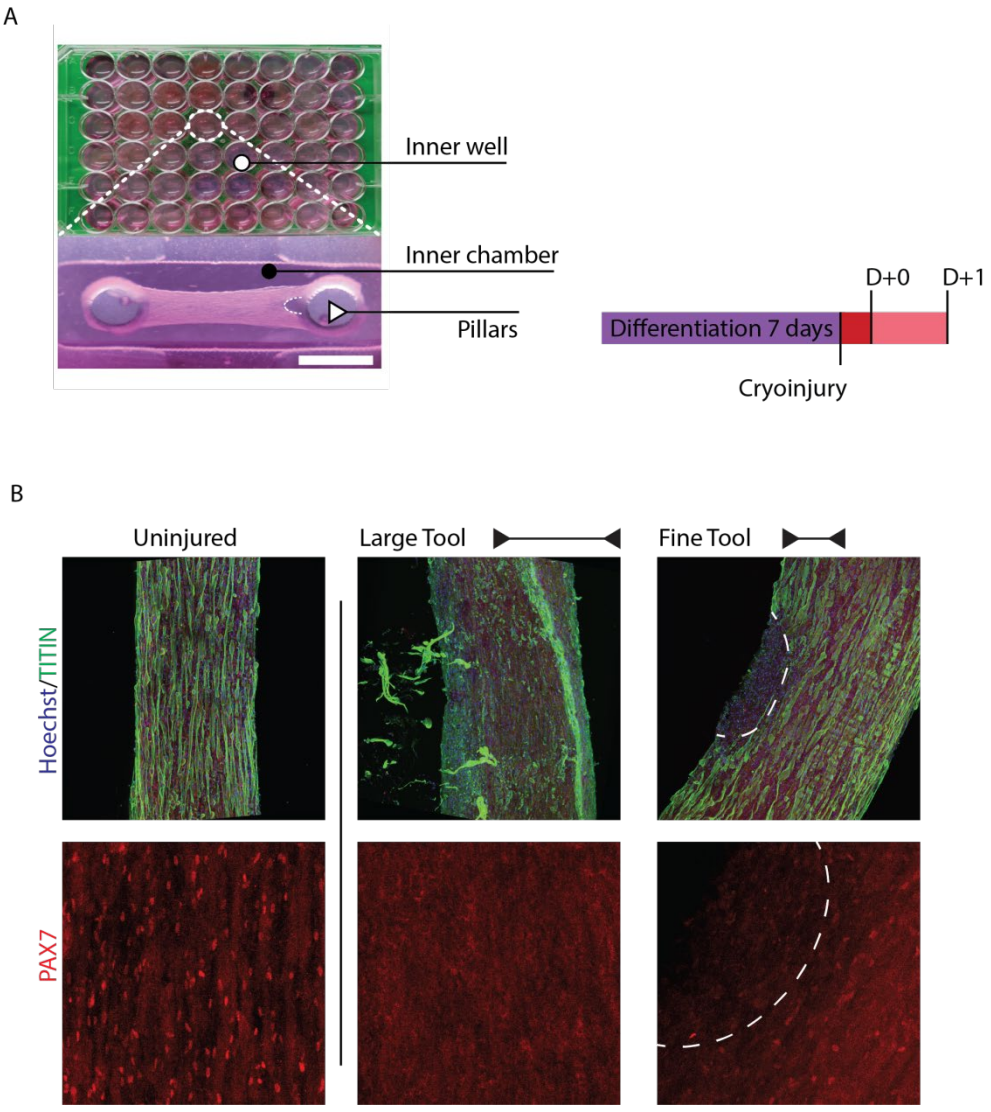


Figure 1. Effect of local cryoinjury on muscle fibers and SCs. *(A) 3D printed platforms are placed in 24 well plates, differentiated for 7 days, injured and left to recover for one day before analysis. (B) Immunofluorescent analysis of engineered muscles that were injured using cryoprobes of different sizes. Large (3mm), fine (0.8mm). Green TITIN, red PAX7, blue Hoechst.*

Injured TESMs with chemical BaCl2 results in muscle fiber degeneration and mildly altered muscle stem cell fraction

$BaCl_2$ is an often used chemical agent to generate muscle injury *in vivo*. It is considered as a mild injury agent in comparison to cryo or myotoxin induced injury. $BaCl_2$ induces depolarization of muscle fibers, eventually destroying the structure of cell membranes. $BaCl_2$ crystals are easily dissolved in water and can be administered intramuscularly *in vivo*. To avoid adding additional muscle damage via injection, TESMs were chemically injured by exposing muscles to different concentrations of $BaCl_2$ (50µl/ml) mixed in differentiation medium for 6 hours and analysed one day later (figure 2A). Chemically injured TESMs resulted in gradual TITIN+ muscle fiber degeneration. Short TITIN+ muscle fibers were present when using 12% $BaCl_2$ or higher, resulting in small patches of uninjured TITIN+ muscle fibers. While muscle fiber degeneration was visible at high concentrations, no major impact was detected on the PAX7+ cell fraction (figure 2B) unlike *in vivo* studies[18]. Notably, the high solubility of $BaCl_2$ in water was severely reduced when mixed in salt-containing solutions such as cell culture media. We could observe an increasing amount of $BaCl_2$ precipitates in the cell culture plates of injured TESMs when increasing doses of $BaCl_2$ were used (data not shown). These results provide evidence that $BaCl_2$ injuries can exert a mild muscle degeneration while leaving the PAX7+ fraction unaltered in human TESMs. However, the impact of muscle damage can be significantly influenced by its solubility, which could profoundly affect follow-up regenerative studies.

High doses of cardiotoxin lead to major muscle fiber degeneration and PAX7+ cell alteration in engineered muscles

Cardiotoxin is a peptide extracted from snake venoms (*Naja pallida*), commonly used to study muscle injury and regeneration. CTX is a protein kinase C inhibitor which induces the depolarization of muscle cells resulting in membrane disruption. To test the effect of increasing doses of CTX, TESMs were exposed to CTX for 6 hours and analysed one day later (figure 3A). Unlike $BaCl_2$, CTX was highly soluble in the cell culture media used to differentiate the TESMs. Injured TESMs showed increasing muscle degeneration depicted as shortening or total loss of viable

TITIN+ muscle fibers and death of PAX7+ cells. We then determined which dose of CTX was sufficient to induce muscle injury in TESMs. To this end, we quantified the mean TITIN+ myofiber area of injured TESMs and measured whether the pool of PAX7 cells was significantly altered (figure 3B, C). We then determined which dose of CTX was sufficient to induce muscle injury in TESMs. To this end, we-

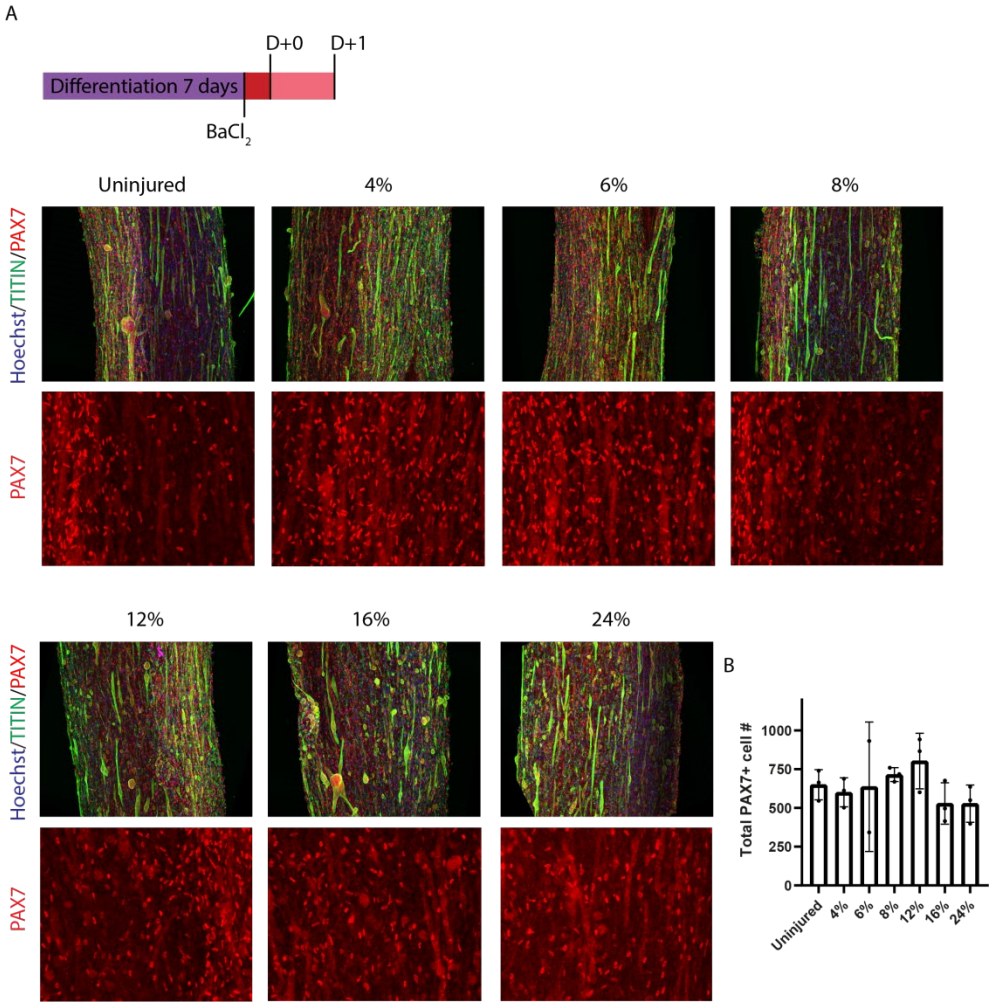


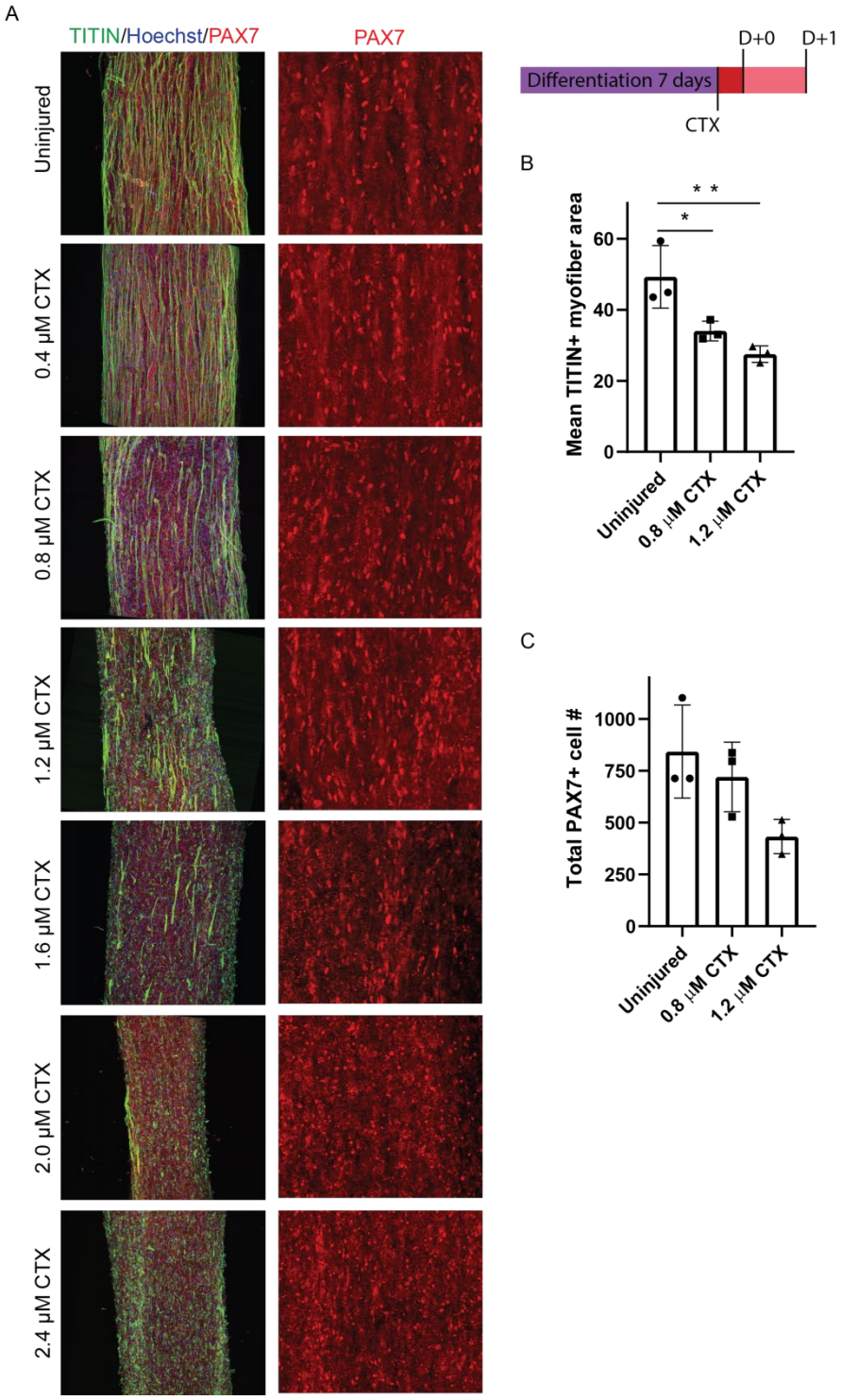
Figure 2. $BaCl_2$ injured TESMs result in TITIN+ muscle fiber degeneration while leaving the PAX7+ cell pool intact. *(A) Immunofluorescence of injured TESMs using increasing doses of $BaCl_2$. Green TITIN, red PAX7, blue Hoechst. (B) Total PAX7 cell quantification in $BaCl_2$ injured muscles. Data are means ± standard error of three biological replicates. ns = not significant, * p < 0.05, **p <0.01 and ***p < 0.001.*

measured whether the pool of PAX7 cells was significantly altered (figure 3B, C). A significant decrease in mean TITIN+ myofiber area was only achieved when using doses equal or higher than 0.8μM of CTX. Increasing doses of CTX also influenced the number of cells expressing PAX7 in TESMs. While using 0.8μM CTX minimally influenced the PAX7+ pool, higher doses had an increasing impact, which could eventually affect muscle regeneration. These results evidence the profound detrimental impact of myotoxins in human engineered skeletal muscles and demonstrate a robust method to exert injury *in vitro* unlike $BaCl_2$.

CTX injured muscles are unable to regenerate suggesting missing crucial elements for efficient muscle regeneration

Next we investigated whether injured TESMs were able to regenerate after injury. To this end, we injured muscles using 0.8μM (mild injury) and 1.2μM (severe injury) CTX, and analysed muscle fiber repair at 7 and 14 days post-injury (figure 4A). We found that engineered muscles were unable to recapitulate the structure and composition of uninjured TESMs. Notably, injured muscles with 1.2μM CTX shrinked when analysed at 7 and 14 days post-injury. In addition, the PAX7 cell pool seemed to be drastically affected, specially after 14 days post-injury. We then quantified the mean TITIN+ area and the number of cells expressing PAX7+ to determine the degree of degeneration (figure 4B, C). While the mean TITIN+ myofiber area equally followed a more severe degeneration according to the CTX dose used, the PAX7+ fraction was profoundly diminished. Remarkably, the PAX7+ fraction remained unaltered at 7 days post-injury in 0.8μM CTX-injured TESMs, but it diminished 3 fold after 14 days post-injury. These results indicate that TESMs derived from purified myogenic progenitors did not induce muscle regeneration upon CTX injury.

Figure 3. CTX injured TESMs result in major TITIN+ muscle fiber degeneration and minor PAX7+ cell ablation. *(A) Immunofluorescence of injured TESMs using increasing doses of CTX. Green TITIN, red PAX7, blue Hoechst. (B) TITIN+ myofiber area after CTX exposure. (C) Total PAX7 cell quantification in injured muscles. Data are means ± standard error of three biological replicates. ns = not significant, * p < 0.05, **p <0.01 and ***p < 0.001.*


A
TITIN/Hoechst/PAX7
PAX7
Uninjured
0.4 µM CTX
0.8 µM CTX
1.2 µM CTX
1.6 µM CTX
2.0 µM CTX
2.4 µM CTX
D+0
D+1
Differentiation 7 days
CTX
B
Mean TITIN+ myofiber area
60
40
20
0
* *
*
Uninjured
0.8 µM CTX
1.2 µM CTX
C
Total PAX7+ cell #
1000
750
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250
0
Uninjured
0.8 µM CTX
1.2 µM CTX

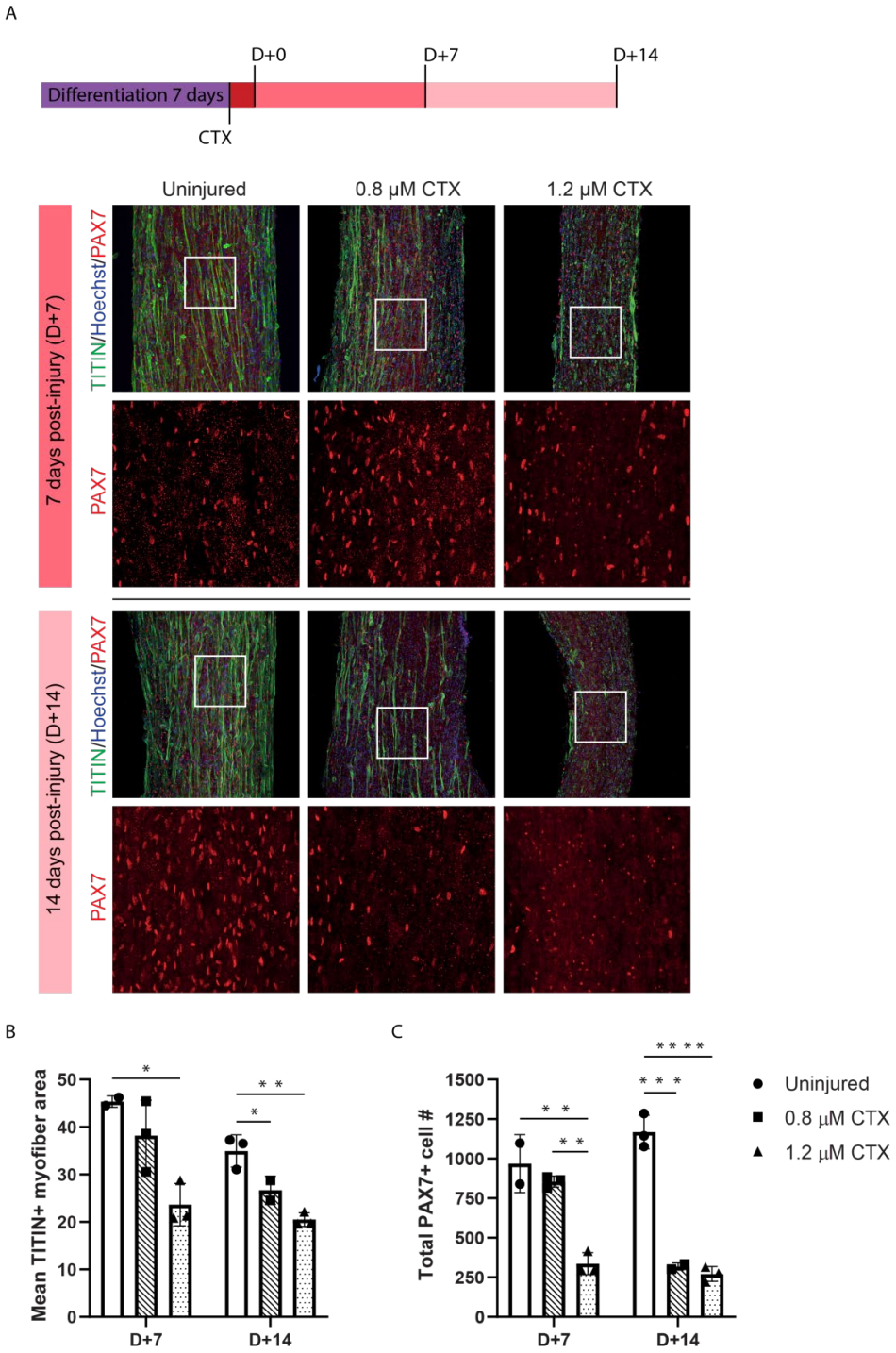
A
D+0
D+7
D+14
Differentiation 7 days
CTX
Uninjured
0.8 µM CTX
1.2 µM CTX
7 days post-injury (D+7)
14 days post-injury (D+14)
TITIN/Hoechst/PAX7
PAX7
B
Mean TITIN+ myofiber area
50
40
30
20
10
0
D+7
D+14
C
Total PAX7+ cell #
1500
1250
1000
750
500
250
0
D+7
D+14
Uninjured
0.8 µM CTX
1.2 µM CTX

Figure 4. Lack of regenerative response of tissue engineered muscles derived from purified myogenic progenitors. *(A) Immunofluorescence of CTX injured TESMs left to regenerate for 7 and 14 days. Green TITIN, red PAX7, blue Hoechst. (B) TITIN+ myofiber area during regeneration. (C) Total PAX7 cell quantification in regenerating muscles. Data are means ± standard error of three biological replicates. ns = not significant, * p < 0.05, **p <0.01 and ***p < 0.001.*

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DISCUSSION

How satellite cells regenerate injured muscle tissue has been mainly investigated using animal models. Recent results have been obtained by the generation of human engineered muscle models for biomedical research. Many of these primarily utilize human muscle cells isolated from healthy donors[13–15,23–25]. However, the limited expansion capacity of primary muscle cell lines makes these a suboptimal cell source for repeated studies using the same donor. The strong expansion and differentiation potential of iPSCs allows efficient generation of skeletal muscle cells for tissue-engineering. Only two studies have previously developed human

engineered muscle models derived from iPSCs[11,12]. However, these used overexpression methods to differentiate iPSCs into skeletal muscle cells which could significantly influence downstream analyses. Additionally, none of these investigated the regenerative responses of injured skeletal muscle models. Here, we used physiologically relevant models of skeletal muscle tissue to investigate human muscle regeneration *in vitro*. For the first time, we show large number of PAX7+ cells present in TESMs derived from human iPSCs using a directed differentiation strategy. In contrast to 2D cultures where SCs are lost within few passages[26,27], here we demonstrate that PAX7+ cells can be maintained for longer time periods in culture. Consequently, engineered muscles could be exploited for the expansion of SCs for cell transplantation therapies in the future. In addition, we evaluate for the first time injury responses in humanized muscle models damaged using multiple injury methods. These strategies could open the door for a better understanding of human skeletal muscle biology in health and disease.

We investigated the effect of three different injury strategies which have been widely used to study muscle injury in mice. Cryodamage is characterized by an acute, diffusive necrosis within the muscle tissue with a strong detrimental effect to both muscle fibers and satellite cells[18]. Muscle regeneration spans from the remaining viable tissue towards the damaged area. It has been studied in the past as an efficient preconditioning regime for muscle cell transplantation which could be used in the future for cell therapy[21,28,29]. However, the effects of cryoinjury in human muscle tissue have not been studied in depth because of the lack of appropriate models of the skeletal muscle tissue. To our knowledge, there are no studies where cryodamage was studied in human muscle models *in vitro*. The severity of cryodamage in mouse skeletal muscle tissue *in vivo* is influenced by the time of cryoprobe exposure and the number of freeze-thaw rounds performed in the same tissue. Similar to animal studies, cryolesions in human TESMs exerted a severe detrimental effect. In contrast to mouse studies, one single exposure to the cryoprobe for a few seconds was sufficient to severely destroy the muscle fibers and muscle stem cells present within the tissue. To allow undamaged muscle tissue to regenerate the injured areas we developed a strategy to minimize the lesion locally to a small area by reducing the size of the probe. These results could

help researchers to further investigate how skeletal muscles respond to severe injuries at extremely low temperatures and find compounds that could accelerate tissue regeneration.

Chemical injury using $BaCl_2$, instead, induced only mild degeneration of muscle fibers while leaving the stem cell pool intact. These results are in line with mouse studies where chemical injuries moderately destroy both the muscle tissue and the SCs[18]. A recent study by Fleming and colleagues showed that $BaCl_2$ injured humanized engineered muscle tissues restored contractile activity a few days after damage[25]. These engineered muscles were made of primary muscle tissue which could potentially contain other cell types that influenced the results. In addition, the low solubility that we observed at similar concentrations raise doubts concerning the actual $BaCl_2$ concentration required to induce a regenerative response. It would be necessary to find ways to efficiently dissolve $BaCl_2$ crystals in cell culture media to perform robust analysis on regenerating muscles using chemical agents.

Multiple studies in mice have applied CTX injury to study muscle regeneration[30]. CTX promotes an acute, localized damage on muscle fibers but is less damaging to satellite cells. Here, we tested increasing doses of CTX and observed gradual degenerative response accordingly. Only one study has recently evaluated the effects of CTX injury in humanized engineered muscles to study injury biomarkers secreted by damaged muscle cells[23]. Whether myotoxin-injured human engineered muscles could regenerate has never been evaluated before. We then determined two different doses with varying impact on the degenerative response to investigate the capacity of injured muscles to regenerate. We observed a decline of TITIN+ myofibers and shrinkage of the whole engineered muscle after CTX injury. Additionally, the number of PAX7+ cells present within the muscles dramatically diminished, further indicating lack of SC activation. The severity of muscle degeneration was more acute with higher doses of CTX. Surprisingly, longer recovery periods resulted in more pronounced muscle wasting. Our results indicate that injured TESMs were unable to repair tissue damage and demonstrated a progressive degenerative response over time. Skeletal muscle regeneration largely depends on the capacity of SCs to proliferate and differentiate

to form new fibers. This process is highly influenced by other non-myogenic cell types such as immune, endothelial and fibroblast cells which can affect the viability and regenerative activity of muscle cells[31–33]. The regulation of skeletal muscle regeneration by the immune system has been widely studied in animal models. Particularly, the role of macrophages to regulate a transient inflammatory response is crucial for SC activation and differentiation[31]. These results suggest that other cell types and/or signalling molecules are required for efficient regeneration of injured humanized muscle models *in vitro*. These could be in the form of additional signalling molecules present when muscle damage occurs. For example, multiple growth factors such as FGF are released from the extracellular matrix together with other molecules secreted after acute muscle damage[34]. Here, we cultured TESMs made of pure muscle progenitor cells in differentiation medium after injury, which might benefit from an initial phase of muscle growth before further myogenic differentiation. However, a recent study evaluated the regenerative potential of CTX injured engineered muscles and demonstrated that these were capable to recover contractile force 7 days after injury[9]. In this study, researchers used primary rat muscle cells which contained other cell types such as fibroblasts and macrophages. A different study addressed this issue by incorporating primary macrophages into engineered muscle models derived from rat tissues[35]. This combination allowed efficient muscle regeneration of engineered muscles *in vitro*. Considering that we used fluorescent activated cell sorting to obtain a pure population of skeletal muscle cells from iPSCs, these results suggest that additional cell types could be crucial. Combining engineered muscle models with strategies to generate macrophages from human iPSCs[36] could potentially be used in the future to increase our understanding of muscle tissue repair in human beings. Other research studies have previously combined different cell types (endothelial, motor neurons) to improve the physiological properties of engineered muscle models[24,37,38]. None of them have tried so far to investigate muscle injury in humanized multicellular muscle models *in vitro*.

Our study is the first to investigate muscle response to multiple injury methods using humanized muscle models *in vitro*. For engineered muscle models to efficiently regenerate injured tissue, other cell types such as macrophages or

signalling molecules might be required. These strategies could be use in the future to gain insight into the regenerative properties of human skeletal muscle diseases and to find novel ways to restore muscle function using a regenerative approach.

MATERIALS AND METHODS

Cell culture of muscle cells derived from iPSCs

Muscle cells were expanded in myogenic proliferation medium consisting of DMEM high glucose (Gibco, Waltham, MA) supplemented with 10% fetal bovine serum (Capricorn Scientific), 1% penicillin-streptomycin-glutamine (P/S/G) (Gibco, Waltham, MA), and 100 ng/mL FGF2 (Prepotech, Rocky Hill, NJ) on extracellular matrix-coated dishes (1:200 diluted, Sigma-Aldrich, E6909). For splitting, myogenic progenitors were detached with TrypLe reagent (Gibco, Waltham, MA) diluted 2x with PBS (Gibco, Waltham, MA). For cryopreservation, myogenic progenitors were detached as described above, and after centrifugation the cell pellet was resuspended in myogenic progenitor proliferation medium supplemented with 10% DMSO and 100 ng/mL FGF2. Standard cell culture techniques were used for the freeze and thaw procedure.

Generation of engineered human skeletal muscles

All engineered muscle constructs were generated using 3D PDMS moulds casted from 3D printed Teflon master moulds and placed in 48 well plates as described in[10]. Briefly, PDMS moulds were sterilized in 70% Ethanol for 30 minutes under UV light in tissue-culture hoods. PDMS moulds were coated with 0.2% pluronic (Invitrogen) for at least 1 hour prior use. The hydrogel used was a mixture gel solution composed of bovine fibrinogen (Sigma) dissolved in DMEM high glucose (Final concentration: 2mg/ml), matrigel growth factor reduced (20% v/v, Corning Life Sciences), thrombin from human plasma (Sigma) dissolved in 0.1% BSA in PBS (Final Concentration: 1% v/v, 0.5U/ml), and myogenic proliferation medium (69% v/v). Cells were suspended in myogenic proliferation medium at a concentration of 16 x 10^6 cells/ml and the suspension was then mixed with the gel solution. A volume of 15µl of the cell mixture were immediately transferred to pre-coated PDMS moulds and left to solidify at 37ºC for 30 minutes before adding the

myogenic proliferation medium supplemented with 6-aminocaproic acid (1.5mg/ml, Sigma) and 100 ng/mL FGF2. After 48 hours, myogenic proliferation medium was switched to differentiation medium consisting of DMEM high glucose supplemented with 1% knock-out serum replacement, 1% ITS-X (all Gibco), 1% penicillin-G (Sigma), 1% glutamax (Sigma), and 6-aminocaproic acid (1.5mg/ml, Sigma). Differentiating TESMs were refreshed every two days. TESMs were incubated in 37ºC, 5% CO_2 humidified incubators with rocking (55rpm).

Injury of TESMs

TESMS were differentiated for 7 days before injury in all experiments. Cryoinjury was performed using steel probes (width end: large ~3mm, small ~0.8mm) chilled in liquid nitrogen. To fully expose TESMs, the medium was carefully removed from the well and the inner chamber of the PDMS mould. Then, TESMs were carefully brought in contact with the chilled probes for ~ 5 seconds. Next, pre-warmed differentiation medium was immediately added and TESMs incubated at 37ºC until fixation. Injury with $BaCl_2$ was conducted by first dissolving the $BaCl_2$ crystals in MiliQ water (24% w/v, Sigma). A volume of 50µl from each concentration of $BaCl_2$ tested was mixed per ml of differentiation medium and used per TESM at 37ºC for 6 hours with rocking. TESMs were washed 3x for 5 minutes each using differentiation medium and incubated at 37ºC until fixation. CTX (from *Naja pallida*; Latoxan, France) injury was performed by exposing TESMs to different dilutions of 100µM CTX dissolved in MiliQ for 6 hours with rocking. TESMs were washed 3x for 5 minutes each using differentiation medium and incubated at 37ºC with rocking until fixation.

Immunofluorescence

Engineered muscles were fixed in 4% paraformaldehyde (PFA, Sigma) for 1 hour at RT and then washed in PBS. Whole fixed muscles were first permeabilized/blocked in 0.3% Triton-X, 3% BSA and 0.1% Tween-20 in PBS (all three from Sigma) for 1 hour at RT on agitation. Washing was performed 3x in PBS for 5 minutes each. Muscles were then incubated in primary antibodies for mouse-IgM-TITIN (1:50, 9D 10-s, DSHB) and mouse-IgG1-PAX7 (1:20, DSHB) in 0.1%

Tween-20, 0.1% BSA in PBS for 1 hour at RT. Followed by secondary antibody incubation using anti-mouse-IgM Alexa 488 (1:500, Thermo Fisher) and anti-mouse-IgG1-biotin (1:250, BD Biosciences) in same antibody solution for 45 minutes at RT. Last, streptavidin 594 (1:500, Invitrogen) was used in same antibody solution for 30 minutes at RT. Nuclear staining was performed using Hoechst 33342 (1:15000, Sigma) for 15 minutes at RT. Samples were kept in PBS at 4ºC before imaging. Imaging was performed using a Leica TCS SP5 confocal microscope (Leica, Germany) using 10x magnification.

Statistics

Mean TITIN+ myofiber area and PAX7+ cell number were determined using ImageJ. Results are presented as means ± SD. Statistical significances among the groups were evaluated using GraphPad Prism 6. Multiple groups were analysed with one-way Anova followed by Tukey's *post-hoc* test. P-values < 0.05 were considered significant.

AUTHORS CONTRIBUTION

Project design: P.H-H and W.W.M. P. Injury experiments were performed by P. H-H, B. L and R. N. Data analysis by P. H-H, B. L, and W.W.M. P. All authors contributed tot data interpretation. Writing by P. H-H, and W.W.M. P. Funding, W.W.M. P.

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Chapter 8

GENERAL DISCUSSION

General Discussion

Current preclinical drug testing and biomedical research extensively relies on inappropriate research models for skeletal muscle disease modeling and therapy development. Either because of their limited expansion potential or poor representative capacity of human disease, current research models are not ideal to identify novel drug candidates and study the pathology of human skeletal muscle disorders. In this thesis, we used human iPSCs, skeletal muscle directed differentiation, engineering devices and gene-editing strategies to develop novel models to better investigate human skeletal muscle disease, with a focus on Pompe disease. A detailed description of past and current efforts towards the development of cell therapies and gene-editing interventions for neuromuscular disease treatment was introduced in **Chapter 1**. Here, we provided an in-depth overview of novel advances in precision genome engineering tools and summarized ongoing clinical interventions in **Chapter 2** and **3**. The potential of iPSC-derived cellular models of human skeletal muscle, their enhanced properties versus current models, and their possible use for generating human muscle tissue *in vivo* was investigated in **Chapter 4**. The use of iPSC-derived skeletal muscle cells also provided useful insight into the development of novel treatment options such as antisense oligonucleotides (AONs), investigated in **Chapter 5**. Novel strategies using gene-editing to permanently correct Pompe disease variants could potentially be used as future treatment options and to understand fundamental aspects of disease pathology. These were developed and described in detail in **Chapter 6**. Additionally, we described how novel physiologically relevant skeletal muscle tissues *in vitro* termed “muscle-on-a-chip” models can revolutionize current preclinical research for neuromuscular diseases. Using muscle injury procedures commonly done in mice to study tissue regeneration, we investigated aspects of human skeletal muscle physiology *in vitro*. The methods used to study skeletal muscle injury *in vitro* were introduced in **Chapter 7**. The strategies investigated here may provide the foundation of future treatments to restore muscle function in Pompe disease and other neuromuscular diseases.

DEVELOPMENT OF PHYSIOLOGICALLY RELEVANT MODELS FOR NEUROMUSCULAR RESEARCH

Current models to study neuromuscular disease include primary human muscle cells, immortalized muscle cell lines and animal models. These models are currently the standard for biomedical research and drug development. Skeletal muscle cellular models have been helpful to investigate early aspects of skeletal muscle disease and discover novel therapies. On the other side, animals such as mice, dogs or non-human primates, have been used to gain insight into the mechanisms of skeletal muscle diseases and preclinical drug development. However, many aspects associated with the origin of the cellular model (muscle biopsies, artificial features), study system (2D cell cultures) and animal models used (typically rodents) make them unable to fully replicate human disease pathology. Consequently, this limits further development of effective therapies to treat neuromuscular disorders.

Use of iPSCs and differentiation methods to study early signs of disease pathology and drug discovery

iPSCs have several advantages over current cellular models. They can be generated using minimally invasively obtained tissues or other materials such as skin fibroblasts, blood or urine. They have unlimited expansion potential and can be differentiated into virtually all cell types of the human body (except extra-embryonal). These capacities make iPSCs ideal for large supply of multiple tissue-specific cell types. In this thesis, we optimized a directed differentiation method for the generation of skeletal muscle cells from human iPSCs (see **Chapter 4**)[1]. This transgene-free method mimics the embryonic development of skeletal muscle via defined medium conditions, resulting in more physiologically relevant models than the immortalized cell lines used for disease modeling. Interestingly, these iPSC-derived skeletal muscle cells show greater expansion potential than primary muscle cell lines, representing more suitable cell sources for large screenings and potential cell therapies for muscle disorders. These properties could be explained by their embryonic/fetal genetic signature, where developing muscle cells are more

prone to migrate and proliferate to form new skeletal muscle tissue upon damage than adult myoblasts with a more specialized role in muscle regeneration[2,3]. Other directed differentiation methods that generate skeletal muscle cells from human iPSCs, these do not report great expansion capacities of cell derivatives *in vitro*[4–7]. In contrast, direct reprogramming methods, based on the overexpression of transgenes, show large expansion of cells while the expression of foreign genes remains active[8–11]. Why does our directed differentiation procedure result in skeletal muscle cells with high proliferative capacities? We considered several hypothetical factors. 1) The strategy we optimized was based on the published work of Borchin *et al*.,[12]. This strategy generates skeletal muscle cells in 30 days which is characterized by three steps: exit pluripotency, induction of proliferation and then commitment. This time frame is shorter than other methods using similar reagents. The fraction of cells obtained within this strategy (independently of the strategy used), although possibly more immature than others, might mimic a population of embryonic muscle cells characterized by large expansion and myogenic potential. 2) Similarly, the cell surface markers we used to purify the muscle cell fraction (CMET+, HNK1-) are different than other differentiation strategies. 3) The culture conditions used to expand the purified muscle cells. The addition of 100ng/ml of FGF2 every two days is higher than what others use to expand skeletal muscle cells of any kind. Upon FGF2 removal, muscle cells stop proliferating and start differentiating. It could be possible that the addition of high amounts of FGF2 might promote self-renewal rather than further myogenic commitment. Other growth factors used in different studies include HGF, IGF, FBS, HS and N2 supplements[4,5,13], which might result in varying impact on the cells' properties.

In addition to their proliferative capacity, myogenic cells generated using our protocol are capable of differentiating further into spontaneously contracting myotubes in 2D cultures at any time point during their expansion. These properties make these skeletal muscle cells suitable for large drug screens. Additionally, the versatility of reprogramming cells from different individuals allows using this technology to generate relevant muscle models of multiple neuromuscular disorders. This feature is especially relevant when developing therapies intended to

target human-specific disease-associated variants for a group of patients with this variant. For example, we used these cells to discover novel AONs for the treatment of patients with the IVS1 variant in Pompe disease[14]. In this thesis, we described how iPSC-derived muscle cells can be used for the screening of novel AONs, with a focus on Pompe disease (see **Chapter 5**). Primary human myoblasts can be expanded for few passages over a period of 2 weeks in culture. This expansion results in an approximated total of 6 x 10^8 myoblasts which is insufficient to conduct large drug screens from a single biopsie[15]. Our iPSC-derived skeletal muscle cells can be expanded to a total of 2 x 10^{16}, greatly increasing their suitability for drug testing and also for the potential future development of cell-based regenerative therapy[1].

Generating isogenic cellular models for robust disease modeling

With the advent of precision gene-editing tools, novel research models carrying single genomic alterations allow the generation of genetically matched controls of human disease. iPSCs are amenable to gene-editing, allowing the generation of isogenic models to diminish the variation when studying lines from multiple individuals. In this thesis, we developed two different gene-correction strategies. 1) Tailored to classic-infantile Pompe disease. 2) Tailored to patients with late onset Pompe disease specifically carrying the IVS1 variant (see **Chapter 6**). By generating isogenic models, we could identify distinct transcriptomic profiles related to metabolism, skeletal muscle development and function. Surprisingly, both forms of Pompe disease showed different alterations related to carbohydrate metabolism. Elevated gene expression was found in metabolic routes involved in aerobic glycolysis such as the TCA cycle and cytosolic glycogen synthesis in classic-infantile Pompe skeletal muscle cells. However, these gene expression changes were not present in IVS1 late onset Pompe disease skeletal muscle cells. Here, we observed down regulation of genes involved in anaerobic glycolysis (from glucose to lactate). We could envision two hypotheses to explain these distinct profiles. 1) These results might be related to the sequence of aberrant events independently of the form of Pompe disease. These gene expression changes might initiate as observed in the late onset Pompe disease cells and ultimately proceed to those

observed in the classic-infantile cells. This might occur at the metabolic level, where affected cells stop utilizing glucose in exchange of other substrates like fatty acids. 2) These results suggest different metabolic compensation mechanisms linked to the enzymatic levels of GAA. If this is true, then the selection of the most effective treatment option could be significantly influenced according to the disease severity. To our knowledge, there is no literature to date that address altered gene expression from monogenetic disorders using isogenic models derived from patients with varying expression levels of the missing gene. In addition to these metabolic differences, the large number of differentially expressed genes observed from a single gene correction is remarkable. These results suggest that large gene expression changes occur early during muscle formation, albeit disease symptoms become often evident much later. It will be of special interest to develop more mature models of skeletal muscle tissue to better evaluate the long-term consequences of the disease. Further research needs to elucidate whether there are druggable targets that benefit the treatment of multiple metabolic myopathies. To address these questions, the generation of patient-derived isogenic lines could be of special relevance. Currently, much effort is still devoted to animal experimentation which often fails to determine human-specific physiological responses in health and disease[16]. Research in animal models has been the preferred research approach in the past years. However, the high variability present when comparing samples from different individuals is one reason why drug responses in animals studies often fail to translate results to human patients[17]. In this thesis it is highlighted that researchers have now the tools to minimize genetic variation and better study aspects of human disease *in vitro*. Overall, iPSC derivatives represent a sustainable source of many different cell types, which are envisaged to be of great value for future disease modeling studies and testing of innovative therapies.

Muscle-on-a-chip disease models to evaluate clinically relevant properties of muscle function *in vitro*

Although current skeletal muscle cell models can provide useful insight into disease pathology and efficacy and toxicity of drugs, the applicability is highly

limited by the culture system used. When differentiating muscle cells are cultured in 2D devices, the contractile properties of these exert a detrimental effect on the longevity as the plate surface is unable to retain the contractile fibers, thus they are lost at an early stage. The development of engineering approaches to generate organ-on-a-chip models from human origin will overcome the limitations of current skeletal muscle models *in vitro*. Hence, the combination of 3D platforms and iPSC-derived skeletal muscle cells resulted in the development of tailored muscle-on-a-chip models[18–20]. These novel models could be used to gain further insight into human disease using more mature muscle tissue and evaluate properties of high clinical relevance such as contractile force. In this thesis, we used muscle-on-a-chip models to investigate regenerative responses of damaged human skeletal muscle *in vitro* (see **Chapter 7**). By applying the same strategies to damage the skeletal muscle used in mice, we could evaluate complex physiological responses in previously unequalled human muscle models, which were impossible to investigate otherwise so far. These models have opened the venue to address unknown aspects regarding the biology of the skeletal muscle in health and disease. Multiple pathologies prominently affect skeletal muscle function. For example, how does aging impact skeletal muscle response to injury? How can features of sarcopenia or cachexia be reverted using small compounds? Some attempts to uncover these questions have been recently made by using TNF-α to induce an aging phenotype in muscle-on-a-chip models[21]. Similarly, combining different cell types, more complex models mimicking pathologies like fibrosis have been recently developed[22]. On this line, others have successfully combined motor neurons and engineered skeletal muscles to allow the modeling of complex disorders like ALS[23]. Advances in the development of physiologically relevant disease models will ultimately improve the successful implementation and pursuing of clinical development of the most promising drugs for human disease. Hence, muscle-on-a-chip models will play a relevant role in the preclinical evaluation of drugs for neuromuscular disorders in the near future.

FUTURE THERAPIES FOR POMPE DISEASE

Pompe disease is one of the few muscle wasting disorders with an available treatment option. ERT was approved in 2006 and greatly improved the prognosis of patients. However, the heterogeneity of response, the potential counteracting effect of antibodies, the high dose and the life-long treatment required, the high costs and the fact that there is still room for improvement are currently encouraging researchers to find alternative treatments. A promising solution lies in the development of novel therapeutics that permanently restore the most crucial physiological activities of affected organs, which is in case of Pompe disease skeletal muscle function. In this regard, cell and gene-editing approaches intended to restore muscle cell function and pathology are being intensely investigated as potential treatments for neuromuscular disorders.

Cell-based therapies using iPSC-derived skeletal muscle cells

To fully restore muscle function, novel treatment options need to efficiently target the muscle stem cell population present in skeletal muscle tissues. Alternatively, healthy/gene-corrected muscle stem cells can be delivered via cell transplantation. In the first attempts to develop muscle cell therapies, myoblasts were used as the initial cell source[24]. Myoblasts however, cannot be sufficiently expanded to target all skeletal muscles of the human body, and more importantly they poorly engraft as satellite cells. Additionally, gene-correction strategies on patient myoblasts are extremely inefficient. Instead, the unlimited expansion capacity of iPSCs and efficient gene-editing outcome make it amenable that these cells represent a more optimal cell type for these therapies. Furthermore, the high proliferative capacity of iPSCs-derived skeletal muscle cells generated using the method described in **Chapter 4** could potentially supply sufficient cells for human muscle cell transplantations. Nowadays, multiple attempts are made to rescue neuromuscular disorders using iPSC-derived skeletal cells (reviewed in[25]). However, *ex vivo* muscle cell therapies are challenged by inefficient engraftment following transplantations in skeletal muscles in men. Most preclinical studies have been performed in mice, however, when tested in non-human primates, the efficiency turned out to be very low (reviewed in[26]). This poor efficacy in animal models more

closely related to humans and the many limitations and considerations to conduct research in non-human primates is greatly limiting the clinical development of cell-based therapies for skeletal muscle diseases. Other challenges involve the capacity of iPSC-derived skeletal muscle cells to engraft as muscle stem cells. In this thesis, we performed several transplantations of dissociated cells administered intramuscularly to pre-injured skeletal muscles of mice. Many of these cells were able to form new muscle fibers but some did not contribute to muscle regeneration and remained in the interstitial space. Similar results were obtained in different studies using multiple methods to differentiate iPSCs into skeletal muscle cells when transplanted in mice[3,5,27]. Further studies will need to elucidate the identity of transplanted muscle cells and find strategies to promote satellite cell engraftment. Muscle cell transplantations are nowadays performed in pre-conditioned skeletal muscles. This is achieved by providing a small injury to the skeletal muscle tissue to be transplanted. Multiple strategies are currently being used for muscle pre-conditioning with varying impact on the engraftment efficiency[28]. Most of them are not suitable for clinical practice such as the use of chemicals, toxins or radiation, which are potentially harmful for human beings. Other potential options such as cryoinjury are being used for other purposes in the clinic such as the treatment of certain malignant tumors (reviewed in[29]). Physical exercise could be a safer alternative, where supervised exercise could induce minor muscle damage and induce a regenerative response that could enhance engraftment efficiency. Ideally, pre-conditioning methods should efficiently modulate skeletal muscle tissues in different ways: 1) they should initiate the regenerative response in the host tissue to allow delivered muscle cells to form new fibers, 2) must be minimally harmful and create an environment in which donor cells efficiently engraft and survive, and 3) should promote a proliferative advantage to the delivered cell fraction. The most suitable pre-conditioning method applied will depend on the inherent skeletal muscle disease of the patient. For example, satellite cells in Duchenne muscular dystrophy undergo proliferative exhaustion due to the constant regenerative response in the affected muscle[30]. Additionally, the impact of a dystrophic environment may have a profound detrimental effect on affected satellite cells[31]. Consequently, under these conditions the endogenous satellite cells have a

reduced capacity to repopulate the skeletal muscle and a mild pre-conditioning regime (like bupivacaine treatment or exercise-induced damage) might be sufficient to achieve efficient cell engraftment. In Pompe disease, recent studies determined that satellite cells of mice affected with Pompe disease are equally capable to regenerate injured muscle than those of wild type mice[32,33]. Considering that these pre-conditioning regimes can induce SC activation in both wild type and Pompe disease mice, an optimal pre-conditioning regime for Pompe disease should aim to diminish or prevent the activation of the endogenous stem cell population prior to cell transplantation. To further investigate the most suitable pre-conditioning methods for efficient muscle cell transplantations, human muscle-on-a-chip models could play a significant role in the future. In **Chapter 7**, we investigated injury methods which are commonly used as pre-conditioning procedures in animal models to allow engraftment of delivered cells in the skeletal muscle tissue. Taking a step forward, muscles injured *in vitro* could serve as a platform to investigate novel strategies to increase engraftment efficiency of transplanted muscle cells. Special attention is required for developing models that closely mimic the dystrophic environment observed in skeletal muscle diseases (fibrosis, fatty acid infiltration) which could influence the cell transplantation efficiency. Alternatively, engineered muscles could potentially be used as transplantable tissue in future. Because of their better capacity to mimic native muscle stem cell conditions, these muscle-on-a-chip models could be an ideal source of quiescent satellite cells. We observed that engineered muscles contained a large number of cells expressing PAX7, indicating a potential cell fraction with muscle regenerative properties. Several studies indicated the high engraftment potential of quiescent satellite cells[34,35]. However, freshly isolated satellite cells rapidly become activated when cultured in plastic surfaces and subsequently lose their stem cell properties and engraftment potential[36]. Many attempts have been made to allow satellite cells to be expanded *ex vivo* by reproducing their niche conditions within the skeletal muscle tissue[37–39]. The natural environment reproduced by these engineered muscles could promote the generation of highly engraftable muscle stem cells for cell therapy. Several options could be envisioned to improve the engraftment capacities of iPSC-derived muscle cells: 1) dissociation of engineered muscle

tissues, isolation of the stem cell fraction and transplantation via injection in preselected muscles[36]; 2) transplantation of whole engineered muscle tissues via surgery[40]; 3) partial dissociation of engineered muscle tissues, isolation of intact myofibers with satellite cells associated to them and delivery via injection in preselected muscles[35]. The first option has been traditionally used for muscle cell transplantations, but it requires extensive tissue manipulation and further dissociation of the muscle tissue which could alter the regenerative properties of the isolated fraction. The second option implicates an invasive method for the delivery of cells that is not really attractive when considering the large size of each individual skeletal muscle. However, in future this option could be especially relevant for patients with volumetric tissue loss. The last option involves a less damaging procedure of isolation where satellite cells remain in their niche conditions (and thus inactivated) and could potentially be used for a less invasive delivery of highly engraftable cells. However, this last option requires PAX7 expressing cells to fully mature into muscle stem cells with high regenerative properties as adult satellite cells present *in vivo*. Overall, these models could greatly accelerate the development of efficient therapies to reverse skeletal muscle wasting via efficient cell therapies.

Gene-editing strategies for classic-infantile and late onset forms

Permanent correction of human disease-associated variants through precision gene-editing is revolutionizing biomedical research. Gene-editing tools such as CRISPR/Cas are nowadays widely used to develop novel therapeutic strategies for genetic disorders of all kinds. Multiple variations of CRISPR/Cas systems with modified properties that increase their specificity and versatility are being developed. Additionally, multiple delivery vehicles to efficiently carry the gene-editing components to target tissues/organs are accelerating its clinical applicability. In this thesis, we reviewed novel advancements towards safe and efficient gene-editing tools and current clinical practice for the treatment of human disease (see **Chapter 2** and **3**)[41,42]. Precision genome medicine is rapidly progressing for the treatment of monogenetic disorders due to the feasibility of conducting single genome modifications. Different delivery approaches are being

investigated for skeletal muscle disorders. Most research is devoted to *in vivo* gene-editing via the systemic administration of AAV vehicles to reach all skeletal muscle organs and heart tissues. Other delivery vehicles used for skeletal muscle gene correction include nanoparticles and lipid particles among others described in **Chapter 2**. However, *in vivo* gene-editing strategies could pose serious risks of prolonged unintended DNA variants difficult to monitor prior to its administration. Additionally, they should efficiently target the satellite cell population to ensure long-term reversal of skeletal muscle wasting. Yet, few studies have attempted to determine the editing efficiency of satellite cells using AAVs[43]. On the other hand, *ex vivo* gene-editing allows for efficient monitoring of off-target effects and thus increase the safety of the intervention. This has promoted the first clinical trials for the treatment of blood disorders, viral infection diseases and several cancer types as described in **Chapter 3**. Nevertheless, *ex vivo* gene-editing requires an efficient engraftment of modified cells (like satellite cells) which remains challenging for the skeletal muscle. No research has been published so far on *in vivo* gene-editing, as a potential treatment option for Pompe disease. Instead, extensive efforts have been conducted to develop traditional gene therapies using AAV vectors targeting the liver for normal or optimized secretable GAA (reviewed in[44]) or directly into the muscle[45]. However, AAVs need to ensure prolonged gene expression. AAVs have minimal risk of transgene insertion into the patient's genome, but recent serious adverse events even resulting in death have been reported following very high dosage of liver targeting AAV for X-linked myotubular myopathy. Additionally, a large part of the general population is immunized against these vectors, because they experienced common human AAV virus infections during their life, which could significantly limit its use in patients as delivery vehicles (reviewed in[46]). Alternatively, *ex vivo* gene-editing could potentially overcome many of the limitations of delivering the gene-editing components directly into skeletal muscle tissue. In this thesis, we develop two different gene-editing strategies for Pompe disease. One generic approach we investigated for the classic-infantile form and one to correct the IVS1 variation in late onset Pompe disease (see **Chapter 6**). To restore GAA enzyme activity in cells of classic-infantile Pompe patients, we inserted a cDNA copy of the *GAA* gene into the *AAVS1* locus (see **Chapter 4**)[1].

This approach could be used in any genetic background due to the high homology of this locus across different individuals and open chromatin conformation to ensure long-term transgene expression[47]. Hence, this approach could also restore GAA expression in the late onset form and the principle could be used to restore missing gene expression in multiple monogenetic disorders. However, special attention must be taken to the selection of the promoter driving transgene expression, as it has been recently shown that potential silencing effects may occur depending on the promoter used[48]. To study promoter silencing at the *AAVS1* locus in skeletal muscle tissue, research studies could potentially use the muscle-on-a-chip models we developed which can be maintained for longer periods in culture than differentiating muscle cells in regular 2D systems. Knock-in gene-editing strategies such as described above for the *AAVS1* locus are, however, less efficient compared to genomic deletions[41]. Strategies intended to restore endogenous expression of altered genes via gene reframing though DNA deletions are rapidly progressing into the clinic[42]. We could increase the endogenous expression of *GAA* by removing a large region within the intron 1 in iPSCs from IVS1 Pompe cells. This gene-editing strategy proved to be efficient in multiple patient lines. These two gene-editing strategies combined with our method to generate pure, expandable skeletal muscle cells could potentially be used in the future as part of autologous cell therapies. To evaluate the safety of these interventions, it remains to be studied whether off-target effects potentially caused by natural Cas9 nucleases when targeting these genomic locations could exist. These could be solved by using alternative nucleases with improved sensitivity. Safe and efficacious gene-editing interventions will further require additional studies in animal models to fully determine their therapeutic potential before entering into human use.

CONCLUSION

With the advent of new precision genome engineering platforms and cell therapies, the development of novel treatment options to permanently correct human diseases is a step closer to standard medical practice. The use of gene-editing to specifically introduce desired genome changes and stem cell differentiation

methods coupled with engineering techniques are improving the development of physiologically relevant tissues from human origin for biomedical research. Novel human derived (disease) models resembling affected tissues/organs could enormously improve current preclinical evaluation of drugs and thus, increase the success of drug candidates in clinical development. Further research into humanized *in vitro* models, efficient muscle cell transplantations and safe gene-editing interventions will greatly impact human healthcare in the future.

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Addendum

SUMMARY

Pompe disease is a severe metabolic myopathy caused by disease-associated variants in the acid alpha-glucosidase (*GAA*) gene resulting in intralysosomal accumulation of glycogen. Patients benefit from enzyme replacement therapy (ERT) which consists of intravenously applied recombinant human GAA enzyme. ERT greatly improves the prognosis of Pompe disease patients. However, the variation in treatment outcome, the high dose and life long weekly/biweekly infusions required, and the high costs encourage researchers to improve the understanding of skeletal muscle disease and search for alternative and next generation treatment options. This thesis includes studies on the molecular mechanisms inducing skeletal muscle pathology in Pompe disease and elaborates on novel treatment options based on gene-editing tools and antisense oligonucleotides using human induced pluripotent stem cells (hiPSCs).

In **Chapter 1**, we provide a clinical description of Pompe disease and a historical overview of ERT and the current limitations to fully restore muscle function. We also describe the remarkable capacity of satellite cells (SCs) to repair the skeletal muscle tissue upon damage in healthy conditions and in Pompe disease. We provide an overview of current limitations of the use of satellite cells for cell therapy and the capacity of hiPSCs as an unlimited source of skeletal muscle cells. We continue with a description of current precision gene-editing strategies for the treatment of neuromuscular diseases. We end this chapter with a brief introduction on the potential of tissue engineering techniques to develop physiologically relevant models of skeletal muscle tissue to be used for disease modelling and drug development. In **Chapter 2**, we provide a detailed description of up-to-date technological advances towards safe, efficient gene-editing tools and delivery strategies for the treatment of human disease. Natural CRISPR/Cas variants are known to generate genome-wide off-target effects which could pause its translation to the clinic. This chapter provides an overview of innovative molecular engineering strategies to improve specificity and efficiency of gene-editing tools for the permanent correction of disease-associated variants. In **Chapter 3**, we elaborate on current clinical interventions using precision gene-

editing tools such as zinc fingers, TALENs and CRISPR/Cas platforms. Current clinical trials applying infusions with *ex vivo* genetically-corrected cells are ongoing (or under development) for patients with cancer (e.g. immunotherapy such CAR-T cells), viral infections (e.g. HIV), hematological disorders (e.g. β-thalassemia) and metabolic disorders (e.g. muccopolysaccharidoses). More recently, *in vivo* gene-editing clinical studies have started for the treatment of Leber's congenital amaurosis, which is amenable to this approach due to the tolerogenic capacities of the eye. We also provide an overview of current challenges for the implementation of gene-editing platforms in the clinic. In **Chapter 4**, we describe in detail the generation of skeletal muscle cells from human iPSCs based on a directed differentiation method and further characterized their expansion and differentiation potential. Here, we utilized 15 donor cells lines (from healthy donors and patients with Pompe disease) and determined that their expansion potential was up to 5 x 10^{11} fold. We demonstrated that these muscle progenitors had a high recovery after thawing and differentiated further into contractile myotubes in culture. Additionally, we describe a gene-editing strategy to restore *GAA* expression in skeletal muscle cells derived from classic infantile Pompe disease patients leading to glycogen clearance. Lastly, we demonstrated that myogenic cell transplantation in pre-injured muscles of mice could partially contribute to muscle regeneration, opening the door for future regenerative therapies for muscle disorders. In **Chapter 5**, we provide a detailed strategy to evaluate therapeutic correction of gene expression in patient skeletal muscle cells using antisense oligonucleotides (AONs). AONs have been widely studied as therapeutic agents to correct aberrant splicing and increase endogenous expression of genes. However, skeletal muscle cells have a poor uptake of AONs whose efficiency is linked to their chemical backbone. Here, we describe a method to efficiently deliver PMO modified AONs to skeletal muscle cells suitable for drug discovery. In **Chapter 6**, we developed a novel gene-editing strategy to correct the common childhood/adult Pompe c.32-13T>G splicing variant (IVS1) and restore endogenous *GAA* expression. Shortening *GAA* intron 1 corrected aberrant splicing and increased GAA activity in patient derived myotubes. This strategy could potentially be used in the future for the permanent correction of the IVS1 variant and to restore muscle function in

childhood/adult Pompe disease. In this study, we conducted transcriptomic analyses of isogenic myotubes derived from IVS1 childhood/adult onset patients and classic infantile patients to search for early gene expression changes. We found distinct transcriptomic profiles for each form of Pompe disease and which implicated genes are involved in glucose metabolism, muscle contraction and development. These results indicate altered cellular metabolic functions preceding evident muscle pathology in Pompe disease. In **Chapter 7**, we studied skeletal muscle response to injury in humanized engineered muscle models *in vitro*. Here, we used 3 dimensional (3D) models of skeletal muscle tissue derived from hiPSCs to study injury and regeneration. We observed distinct degenerative responses according to the injury method used: cryodamage, chemical and myotoxin injury. While cryodamage was the most severe, chemical and myotoxin injuries were less detrimental to the SC population. We then observed a lack of regenerative response from myotoxin injured muscle models which resulted in progressive degeneration of muscle fibers and SCs. Since skeletal muscle repair (such as SC activation, survival and differentiation) is regulated by other non-myogenic cell types (such as immune, fibroblast and endothelial cells), these results offer a 'clean' *in vitro* system for the study of additional signaling molecules and/or cell types that are required for efficient muscle repair. In **Chapter 8**, we highlight the impact of these findings in the context of searching for alternative treatment options for neuromuscular diseases and novel models to improve our understanding of disease pathology, especially for Pompe disease.

SAMENVATTING

De ziekte van Pompe is een ernstige metabole myopathie veroorzaakt door ziekte-geassocieerde varianten in het gen voor zure alfa-glucosidase (*GAA*), wat resulteert in intralysosomale ophoping van glycogeen. Patiënten hebben baat bij enzymvervangende therapie (ERT), dat bestaat uit recombinant humaan GAA wat intraveneus wordt toegediend. ERT verbetert de levensverwachting van patiënten met de ziekte van Pompe aanzienlijk. Echter, de variatie in de uitkomst van de behandeling, de hoge dosis en levenslange (twee)wekelijkse infusies die nodig zijn, en de hoge kosten moedigen onderzoekers aan om het begrip van deze ziekte te verbeteren en te zoeken naar alternatieve behandelingsopties. Dit proefschrift omvat onderzoeken naar de moleculaire mechanismen die skeletspierpathologie induceren bij de ziekte van Pompe en gaat in op nieuwe behandelingsopties op basis van genetische correctie en antisense oligonucleotiden waarbij gebruik gemaakt wordt van humane geïnduceerde pluripotente stamcellen (hiPSCs).

In **hoofdstuk 1** geven we een klinische beschrijving van de ziekte van Pompe en een historisch overzicht van ERT waarin ook de huidige beperkingen om de spierfunctie volledig te herstellen worden besproken. We beschrijven het vermogen van satellietcellen (SCs) om het skeletspierweefsel te herstellen bij spierschade onder normale omstandigheden en bij de ziekte van Pompe. Daarnaast bieden we een overzicht van de huidige beperkingen van het gebruik van satellietcellen voor celtherapie en de capaciteit van hiPSCs als een onuitputtelijke bron van skeletspiercellen. We gaan verder met een beschrijving van de huidige precisie genbewerkingsstrategieën voor de behandeling van neuromusculaire ziekten. We sluiten dit hoofdstuk af met een korte inleiding over de mogelijkheden van het maken van weefsels in het lab om fysiologisch relevante modellen van skeletspierweefsel te ontwikkelen die gebruikt kunnen worden voor het nabootsen van verschillende ziektes en de ontwikkeling van medicijnen. In **hoofdstuk 2** geven we een gedetailleerde beschrijving van actuele technologische vooruitgang richting veilige en efficiënte technieken om genetische aanpassingen te bewerkstellen en de strategieën waarmee deze kunnen worden afgeleverd aan

de cellen, zodat dit gebruikt zou kunnen worden voor de behandeling van menselijke ziekten. Van natuurlijke CRISPR/Cas-varianten is bekend dat ze over het gehele genoom aspecifieke effecten kunnen veroorzaken die de vertaling naar de kliniek kunnen belemmeren. Dit hoofdstuk biedt een overzicht van innovatieve moleculaire strategieën om de specificiteit en efficiëntie van de precisie genbewerkingsstrategieën voor de permanente correctie van ziektegerelateerde varianten te verbeteren. In **hoofdstuk 3** gaan we dieper in op de huidige klinische interventies met behulp van precisie genbewerkingsstrategieën zoals zinkvingers, TALEN's en CRISPR/Cas-platforms. Op dit moment zijn er klinische studies gaande waarin gebruik gemaakt wordt van infusies met in the laboratorium (ex *vivo*) genetisch gecorrigeerde cellen voor patiënten met bijvoorbeeld kanker (bijv. immunotherapie met behulp van CAR-T-cellen), virale infecties (bijv. HIV), hematologische aandoeningen (bijv. β-thalassemie) en metabole stoornissen (bijv. muccopolysaccharidosen). Meer recentelijk zijn *in vivo* klinische studies met precisie genbewerkingsstrategieën gestart voor de behandeling van Leber's congenitale amaurosis, een aandoening van het oog. We geven daarnaast ook een overzicht van de huidige uitdagingen voor de implementatie van precisie genbewerkingsstrategieën in de kliniek. In **hoofdstuk 4** geven we een gedetailleerde beschrijving van de generatie van skeletspiercellen uit menselijke iPSCs en karakteriseerden we hun eigenschappen verder. Hierbij werd er gebruik gemaakt van 15 donor cellijnen (van gezonde donoren en patiënten met de ziekte van Pompe) en vonden we dat de cellen konder vermeerden tot 5×10^{11} keer zonder verlies van hun spierstanmcel eigenschappen. We toonden aan dat deze voorlopers van spiercellen na het ontdooien functioneel bleven en konden differentiëren tot samentrekkende myotubes, kleine strengen van gefuseerde spiercellen, tijdens de kweek. Bovendien beschrijven we een precisie genbewerkingsstrategieën om *GAA* expressie in skeletspiercellen afkomstig van klassieke infantiele Pompe-patiënten te herstellen waardoor de glycogeenopstapeling in deze cellen wordt verholpen. Ten slotte hebben we aangetoond dat celtransplantatie van menselijke spierstamcellen, verkregen uit iPSCs, in spieren van muizen gedeeltelijk kan bijdragen aan spierregeneratie, waardoor de deur wordt geopend voor toekomstige regeneratieve therapieën voor

spieraandoeningen. In **hoofdstuk 5** geven we een gedetailleerde strategie om therapeutische correctie van genexpressie in skeletspiercellen van patiënten te evalueren met behulp van kleine RNA moleculen genaamd antisense oligonucleotiden (AONs). AONs zijn op grote schaal bestudeerd als therapeutische middelen om afwijkende splicing te corrigeren en de natuurlijke expressie van genen te verhogen. Hier beschrijven we een methode om AONs te testen in skeletspiercellen die geschikt zijn voor het ontdekken van geneesmiddelen. In **hoofdstuk 6** ontwikkelden we een nieuwe genbewerkingsstrategie om een zeer vaak voorkomende *GAA* DNA variant, de c.32-13T>G variant (IVS1), te corrigeren en natuurlijke *GAA*-expressie te herstellen. Het verkorten van *GAA* intron 1 corrigeerde de afwijkende splicing (dit is een knip en plak proces in de cel die het RNA klaarmaakt voor het maken van eiwit) en verhoogde de GAA activiteit. Deze strategie zou in de toekomst mogelijk kunnen worden gebruikt voor de permanente correctie van de IVS1-variant en om de spierfunctie in de ziekte van Pompe te herstellen. In deze studie voerden we transcriptomische analyses (waarin de activiteit van alle genen van het DNA tegelijkertijd wordt gemeten) uit van skeletspiercellen van IVS1-patiënten en klassieke infantiele patiënten om de vroege genexpressieveranderingen te bestuderen. We vonden verschillende transcriptomische profielen voor elke vorm van de ziekte van Pompe en identificeerden genen die betrokken zijn bij glucose metabolisme, spiercontractie en spierontwikkeling. In **hoofdstuk 7** bestudeerden we de reactie van skeletspieren op letsel in menselijke spiermodellen in *vitro*. Hierbij gebruikten we 3 dimensionale (3D) modellen van skeletspierweefsel afgeleid van hiPSCs. We observeerden duidelijke degeneratieve reacties op de gebruikte verwondingsmethode: vriesschade, chemisch en myotoxineletsel. Terwijl vriesschade het ernstigst was, waren chemische en myotoxineletsels minder schadelijk voor de spierstamcel populatie. We zagen een gebrek aan spierherstel van spiermodellen die beschadigd waren met myotoxine. Dit resulteerden in progressieve degeneratie van spiervezels en spierstamcellen. Aangezien skeletspierherstel (zoals stamcelactivatie, overleving en differentiatie) mede wordt gereguleerd door andere celtypen in de spieren, zoals immuun-, fibroblast- en bloedvatcellen, en het huidige model bestond uit zuivere spiercellen, bieden deze

resultaten een 'schoon' *in vitro* systeem voor de studie van aanvullende signaalmoleculen en/of celtypen die nodig zijn voor efficiënt spierherstel. In **hoofdstuk 8** belichten we de implicaties van deze bevindingen in de context van de zoektocht naar alternatieve behandelingsopties voor neuromusculaire ziekten en nieuwe modellen om ons begrip van ziektepathologie te verbeteren, met name voor de ziekte van Pompe.

RESUMEN

La enfermedad de Pompe se describe como una miopatía metabólica severa causada por mutaciones en el gen de la glucosidasa-alfa acida (GAA), cuya deficiencia resulta en la acumulación intralisosomal de glucógeno. Los pacientes afectados se benefician a día de hoy de la terapia de reemplazo enzimático (ERT), que consiste en la administración intravenosa de la enzima GAA recombinante humana. El tratamiento por ERT mejora enormemente el pronóstico de los pacientes de la enfermedad de Pompe. Sin embargo, la heterogeneidad de resultados clínicos tras el tratamiento, las alta dosis e infusiones semanales/bisemanales requeridas, y los altos costos de tratamiento, animan a los investigadores a tratar de entender mejor como se produce la enfermedad en el músculo esquelético y estimulan la búsqueda de tratamientos alternativos de nueva generación. Esta tesis indaga en los mecanismos moleculares que inducen la patología de la enfermedad de Pompe en el musculo esquelético, e investiga nuevos tratamientos basados en la edición genética y los oligonucleótidos antisentido (AONs) usando células madre pluripotentes inducidas (hiPSCs).

En el **Capítulo 1**, proporcionamos una descripción clínica de la enfermedad de Pompe y una visión general sobre el tratamiento ERT y las limitaciones actuales para restaurar el normal funcionamiento del músculo esquelético. También describimos la destacada capacidad de las células satélite (SCs, células madre de músculo esquelético) para reparar el tejido muscular tras un daño tisular en condiciones normales y en la enfermedad de Pompe. Proporcionamos una visión general de las limitaciones actuales del uso de las SCs para terapia celular y la capacidad de las hiPSCs como una fuente inagotable de células musculares. Continuamos con una descripción de las estrategias actuales de edición genética y tratamiento de enfermedades neuromusculares. Acabamos este capítulo con una breve introducción del potencial de las técnicas de ingeniería tisular para desarrollar modelos fisiológicamente relevantes de músculo esquelético para el estudio de enfermedades musculares y el desarrollo de fármacos. En el **Capítulo 2**, proporcionamos una descripción detallada de los avances tecnológicos actuales para el desarrollo y administración de técnicas seguras y eficaces de edición

genética para el tratamiento de enfermedades humanas. Las variantes naturales del CRISPR/Cas son conocidas por generar efectos secundarios a nivel genómico que retrasan su plena utilización en el ámbito clínico. Este capítulo proporciona una visión general sobre estrategias innovadores de ingeniería molecular para mejorar la especificidad y la eficiencia de las herramientas de edición genética para la corrección permanente de mutaciones en el genoma. En el **Capítulo 3**, elaboramos sobre las intervenciones clínicas actuales que utilizan plataformas de edición genética tales como los dedos de cinc, TALENs o CRISPR/Cas. Actualmente, estos ensayos clínicos están dominados por trasplantes de células genéticamente editadas *ex vivo* para pacientes de cáncer (ej. Inmunoterapia mediante células CAR-T), infecciones víricas (ej. VIH), desórdenes hematológicos (ej. Beta-talasemia) y desórdenes metabólicos (ej. Mucopolisacaridosis). Más recientemente, estudios clínicos de edición genética *in vivo* han comenzado para el tratamiento de la amaurosis congénita de Leber, el cual es susceptible a este enfoque debido a las capacidades tolerogénicas del ojo. También proporcionamos una visión general de los desafíos actuales para la implementación de terapias de edición genética en el ámbito clínico. En el **Capítulo 4**, describimos en detalle la generación de células de músculo esquelético a partir de hiPSCs, basadas en el método de la diferenciación dirigida y caracterización de su potencial de expansión y diferenciación en células del músculo esquelético. Aquí utilizamos 15 líneas celulares de donantes (desde individuos sanos a pacientes con la enfermedad de Pompe) y determinamos un potencial de expansión celular de 5×10^{11} desde su generación hasta que alcanzan la senescencia celular. Demostramos que estas células musculares poseen una alta recuperación de descongelación (previa congelación en nitrógeno líquido) y de diferenciación en fibras musculares con capacidad de contracción en cultivo. También describimos una nueva estrategia de edición genética, mediante CRISPR/Cas, para restaurar la expresión del gen *GAA* en células musculares derivadas de pacientes con la enfermedad clásico-infantil de Pompe (la más severa de las existentes), cuyo resultado promueve la reducción de glucógeno. Por último, demostramos que el trasplante de estas células en músculos previamente dañados puede contribuir parcialmente a la regeneración muscular en ratones, abriendo una puerta al futuro de las terapias de

regeneración tisular para el tratamiento de las enfermedades del músculo esquelético. En el **Capítulo 5**, proporcionamos una detallada estrategia para evaluar la corrección terapéutica de expresión génica en células musculares de pacientes mediante el uso de AONs. Estos han sido estudiados en gran medida como agentes terapéuticos para la corrección de anomalías en el "splicing" (corte y empalme) de RNA e incrementar la expresión genética de genes cuyos niveles de expresión se ven afectados por mutaciones. Sin embargo, las células musculares se caracterizan por una pobre absorción de estos AONs, cuya eficiencia está íntimamente ligada a su estructura química. Aquí, describimos un método para la administración eficiente de AONs PMO-modificados en células musculares apta para el estudio y desarrollo de nuevos fármacos. En el **Capítulo 6**, desarrollamos una innovadora estrategia de edición genética (mediante CRISPR/Cas) para corregir mutaciones en la forma adolescente/adulta de la enfermedad de Pompe (la menos severa) basada en la mutación de splicing c.32-13T>G (IVS1), y así restaurar la expresión del gen *GAA*. El acortamiento del primer intrón del gen *GAA*, corrige la anomalía en el splicing e incrementa la actividad enzimática en fibras musculares derivadas de pacientes. Esta estrategia puede ser potencialmente utilizada en el futuro para la corrección permanente de la mutación IVS1 y restaurar la función muscular en pacientes con la forma adolescente/adulta de la enfermedad de Pompe. En este estudio, llevamos a cabo análisis transcriptómicos usando fibras musculares isogénicas derivadas de pacientes con la mutación IVS1 de la forma adolescente/adulta y pacientes con la enfermedad clásico-infantil de la enfermedad de Pompe para investigar cambios de expresión génica a escala genómica. Aquí encontramos distintos perfiles transcriptómicos para cada forma de la enfermedad de Pompe cuyos genes implicados se muestran involucrados en el metabolismo de la glucosa, la contracción y el desarrollo del tejido muscular. Estos resultados apuntan a una alteración de las funciones metabólicas celulares previos al desarrollo de la patología muscular presente en la enfermedad de Pompe. En el **Capítulo 7**, estudiamos la respuesta al daño tisular del músculo esquelético utilizando modelos musculares humanizados diseñados mediante ingeniería tisular *in vitro*. Aquí utilizamos modelos en 3 dimensiones (3D) del músculo esquelético derivados de hiPSCs para estudiar la regeneración del tejido

muscular humano. De esta manera, estudiamos distintas respuestas de regeneración tisular de acuerdo al método utilizado para dañar el tejido: por congelación, mediante agentes químicos o mediante toxinas. Mientras que el daño por congelación fue el más severo, los agentes químicos y toxinas fueron menos dañinos, especialmente para las células satélite. Sin embargo, al tratar los modelos musculares con toxinas, observamos una falta de respuesta de regeneración tisular (pese a la presencia de SCs), provocando una degeneración progresiva de las fibras musculares y de la población de SCs. Ya que la reparación del músculo esquelético (tal como la activación de SCs, supervivencia y diferenciación) se regula mediante otras células no musculares (como células del sistema inmune, fibroblastos y células endoteliales), estos resultados ofrecen un sistema *in vitro* "puro" donde estudiar la interacción de otro tipo de células y señales moleculares necesarias para la reparación eficiente del tejido muscular. En el **Capítulo 8**, destacamos el impacto de estos descubrimientos en el contexto de la búsqueda y desarrollo de nuevos tratamientos para enfermedades neuromusculares y el estudio de patologías como la enfermedad de Pompe mediante el uso de modelos innovadores del musculo esquelético.

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Herrero-Hernandez, P. van der Wal, E. in 't Groen, SLM. Kruijt, J. van IJcken, WF. Schaaf, GJ. van der Ploeg, A. Pijnappel, WWM. Shortening of intron 1 restores the c.-32-13T>G *GAA* splicing variant in iPSCs-derived skeletal muscle cells from childhood/adult Pompe patients. *Submitted*

Herrero-Hernandez, P. Los, B. Nazir, R. van der Ploeg, A. Pijnappel, WWM. Engineered human skeletal muscles to study injury and regeneration *in vitro*. *In preparation*

CURRICULUM VITAE

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Professional Experience

PhD Candidate
October 2016 – March 2021
Erasmus Medical Center, Dept Clinical Genetics, Dept Pediatrics, the Netherlands
Center for Lysosomal and Metabolic diseases, Molecular Stem Cell Biology group

"Engineering human iPSC-derived skeletal muscle to model Pompe disease"

Research Intern (MSc)
September 2015 – September 2016
Erasmus Medical Center, Dept Clinical Genetics, Dept Pediatrics, the Netherlands
Center for Lysosomal and Metabolic diseases, Molecular Stem Cell Biology group

"Development of alternative approaches to treat Pompe disease"

November 2014 – July 2015
Erasmus Medical Center, Dept Cell Biology, the Netherlands

"Identification of Sip1/Zeb2 target genes during neuronal differentiation"

Research Intern (BSc)
February 2013 – August 2013
National Institute for Medical Research (MRC), Division of Molecular Neurobiology, United Kingdom

"A neuron stem cell model for studying striatal interneuron specification"

June 2012 – July 2012
National University of Ireland Galway (NUI), Molecular Diagnostics Research group, Ireland

"Bacterial meningitis diagnostic landscape with particular reference to Haemophilus influenzae as a causative agent"

Education

Erasmus University Rotterdam
Doctor of Philosophy (Ph.D) in Biomedical Sciences 2016 - 2021
Master of Science (MSc) in Molecular Medicine 2014 - 2016

Universidad Francisco de Vitoria
Bachelor of Science (BSc) in Biotechnology 2009 - 2013

Athlone Institute of Technology
Bachelor of Science (BSc, ordinary) in Biotechnology 2011 - 2012

Certificates

Entrepreneurship awakening (ECE) – 2020
Venture challenge (NWO) – 2020
Stem cells, organoids and regenerative medicine (EMC) – 2018
Radiation hygiene 5B (EMC) - 2018
Microscopic image analysis (OIC, EMC) – 2017
Laboratory animal science, Art 9. The Netherlands (EMC) - 2017
R-computing (EMC) - 2016
Optical imaging (OIC, EMC) – 2016

PHD PORTFOLIO

	Year	ECTS
Courses		
Laboratory animal science	2017	3
Genetics	2017	3
Safely working in the laboratory	2017	0.5
Microscope image analysis	2018	1
Practical radiation hygiene	2018	1
Stem cells, organoids and regenerative medicine	2018	1
Entrepreneurship awakening	2020	1
Workshops		
MGC PhD Workshop (x3)	2017/2018/201	3
Venture Challenge (NWO)	2020	3
Career support workshop	2020	0.5
National and International Meetings		
Step Forward in Pompe disease meeting, NL	2016	1
International Glycogen Storage Disease congress (GSD), NL	2017	1
11th Dutch Society for Stem Cell Research (DSSCR), NL (x2)	2018/2019	1
European Study Group for Lysosomal Diseases (ESGLD), SPA	2019	1
Society for the Study of Inborn Errors of Metabolism (SSIEM), NL	2019	1
22nd American Society of Gene and Cell Therapy (ASGCT), USA	2019	2
Presentations		
Poster presentation MGC PhD Workshop	2018	0.25
Poster presentation ASGCT	2019	0.25
Oral presentation MGC PhD Workshop	2019	0.5
Oral presentation DSSCR	2019	0.5
Oral presentation SSIEM	2019	0.5
Oral presentation ESGLD	2019	0.5
Investor pitch presentation VC workshop	2020	0.5
Teaching		
Supervisor HBO student internships (x3)	2018/2019/2020	6
Supervisor MSc student literature review	2018	1
Teacher assistant BSc student course “Introduction to scientific literature”	2018	1
Total ECTS		**35**

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