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INVITED REVIEW

Gene therapy strategies in the treatment of hypertrophic cardiomyopathy

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Abstract
Hypertrophic cardiomyopathy (HCM) is an inherited myocardial disease with an estimated prevalence of 1:200 caused by mutations in sarcomeric proteins. It is associated with hypertrophy of the left ventricle, increased interstitial fibrosis, and diastolic dysfunction for heterozygous mutation carriers. Carriers of double heterozygous, compound heterozygous, and homozygous mutations often display more severe forms of cardiomyopathies, ultimately leading to premature death. So far, there is no curative treatment against HCM, as current therapies are focused on symptoms relief by pharmacological intervention and not on the cause of HCM. In the last decade, several strategies have been developed to remove genetic defects, including genome editing, exon skipping, allele-specific silencing, spliceosome-mediated RNA *trans*-splicing, and gene replacement. Most of these technologies have already been tested for efficacy and efficiency in animal- or human-induced pluripotent stem cell models of HCM with promising results. We will summarize recent technological advances and their implication as gene therapy options in HCM with a special focus on treating *MYBPC3* mutations and its potential for being a successful bench to bedside example.

Keywords Hypertrophic cardiomyopathy · Gene therapy · Exon skipping · *trans*-splicing · CRISPR/Cas9 · *MYBPC3* · Gene replacement

Hypertrophic cardiomyopathy

Hypertrophic cardiomyopathy (HCM) is the most common Mendelian-inherited cardiomyopathy and the main cause of sudden cardiac death in individuals below the age of 35, mainly among young athletes (for detailed reviews, see [3, 46]). Its revised estimated prevalence is 1:200 in the general population occurring equally in both genders, whereby recent reports indicate worse survival in women [17, 60]. Clinical manifestations of HCM are variable in terms of disease development, age of onset, and severity of symptoms, causing chest pain, vertigo, syncope, and dyspnea, whereas others patients are in need for early heart transplantation, or die of sudden cardiac death [16, 48, 55]. Causative for these clinical symptoms are structural changes in the heart defined by chaotically oriented cardiac myocytes, so-called myocardial disarray, interstitial fibrosis and hypertrophy, mainly seen in the left ventricle [24]. About 70% of hospitalized HCM patients experience an abnormal thickening of the left ventricle, leading to outflow tract obstruction, which is often associated with normal systolic but impaired diastolic function [49]. If left untreated or undiagnosed, disease progression can result in left ventricular wall thinning, characterized as one of the main risk factors inducing irreversible heart failure and unexpected sudden cardiac death [48]. Current treatment options for HCM are drug-based therapies that aim to relieve associated symptoms and decelerate disease progression, but do not target the genetic cause of HCM (for review, see [70]). Up to now, more than 1400 mutations in over 11 different genes have been identified as a potential cause for HCM (reviewed in [14, 47, 67]). A large majority of these mutations are in genes encoding components of the cardiac sarcomere and are inherited in an autosomal-dominant pattern [64]. Most gene mutations were found in *MYBPC3*, encoding cardiac myosin-binding protein C (cMyBP-C) and in *MYH7*, encoding β-myosin heavy chain (for review, see [10]). Individuals carrying homozygous,

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double or triple mutations have more severe disease progression [18, 25, 28, 58, 75]. Especially, individuals with bi-allelic truncating *MYBPC3* mutations were diagnosed with various forms of cardiomyopathy at birth and quickly developed systolic heart failure and died within the first year [40, 75, 78]. For these infants, there is no curative therapy apart from a heart transplant.

Cardiac gene therapy

The idea of gene therapy as a "therapeutic technique achieving permanent correction of hereditary diseases" has been introduced by Clyde Keeler in 1947 [37]. The first trial of human gene therapy has been approved in 1989 for advanced melanoma [65] and in 2004 for heart failure [30]. Today, more than 2300 clinical trials have been completed, are ongoing, or have been approved worldwide [23].

Probably the biggest hurdle for human gene therapy is sufficient and safe delivery of the gene therapy medicinal product into the patient's body. For gene therapy of cardiac diseases, the currently used viral vectors are adeno-associated virus (AAV). These are small, non-enveloped virus belonging to the *parvoviridae* family with very low to absent pathogenicity in human host. Features such as the ability to transduce terminally differentiated cells (such as cardiomyocytes), absence of integration in the host genome, and long-lasting gene expression made the AAV attractive for gene therapy. In addition, AAVs naturally occur in different serotypes, which exhibit a distinct tissue tropism (for reviews, see [12, 59]). It has been shown that the serotype 9 (AAV9) is the most efficient for cardiac gene transfer after systemic delivery in mouse and large animal models [29, 52, 57, 62, 79]. The successful completion of a SERCA2a gene therapy phase 2 trial demonstrated safety and feasibility of AAV1-mediated gene transfer in humans [31]. Unfortunately, this study failed in showing beneficial outcomes in treated patients [20]. To further limit the expression of the transgene in cardiomyocytes, a cardiomyocyte-specific promoter such as cardiac troponin T could be used. A limitation of AAV-mediated gene therapy is that humans very frequently present neutralizing antibodies against AAV. These neutralizing antibodies might have a dramatic impact on the outcome of gene therapy and is major a problem in case of re-administration. This could be circumvented by pharmacological modulation of the immune response and/or use of another AAV serotype.

Targeting inherited disorders

Specifically for inherited (or monogenic) disease, there has been major progress in the field of molecular-based interventions to suppress the expression of genetic defects on DNA or RNA level, including genome editing, exon skipping, spliceosome-mediated RNA *trans*-splicing, allele-specific silencing, and gene replacement therapy. Tools used are clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, antisense oligonucleotides, RNA interference molecules or wild-type cDNA sequences, often combined with AAV delivery.

Genome editing

CRISPR/Cas9 transformed the field of genome editing by storm. Initially, the CRISPR/Cas9 system was found to be an adaptive immune response in bacteria (for reviews, see [27, 50]). Thirty years later, it has been demonstrated that CRISPR type-2 system proteins, called Cas9, can also function as designer nucleases by associating them with an engineered single guide RNA, which is complementary to a genetic locus of interest [33]. This targeted Cas9 is able to induce a double-strand break that can be resolved by endogenous DNA repair mechanisms such as non-homologous end-joining or by homology-directed repair [13, 38, 45]. Non-homologous end-joining is not desired for gene correction applications because it is prone to introduce additional mutations in the form of insertions and/or deletions. Homology-directed repair corrects the double-strand break site with a supplied exogenous DNA repair template or the non-mutant homologous chromosome, leading to the correction of the mutant allele (Fig. 1a; [42, 77]). Previously used genome editing tools were mostly zinc finger nucleases and transcription activator-like effector nucleases, which proved to be costly and labor intensive (for review, see [26]). CRISPR/Cas9, on the other hand, is a low-priced and quick method for disease modeling in human-induced pluripotent stem cell (hiPSC), mice and larger animal models (for review, see [66]). Recent reports demonstrated successful generation of HCM models or genetic correction in HCM hiPSC with CRISPR/Cas9 genome editing [6, 56, 74].

In the context of human gene therapy, much can be learned from the reports in mice, as viral-based delivery vectors of CRISPR/Cas9 components were able to edit postnatal murine hearts [11, 21, 34]. Targeted delivery of CRISPR/Cas9 components, no adverse side effects and efficient genome editing of non-dividing cardiomyocytes are three necessities that need to be ensured before applying this treatment to humans. Additionally, these studies showed that Cas9 overexpression in cardiomyocytes does not alter baseline cardiac function. In general, editing efficiencies and mosaicism are still limitations when using CRISPR/Cas9 in vivo. Indeed, homology-directed repair occurs primarily during the S/G2 phase of the cell cycle and therefore limits its application in (adult) cardiomyocytes. However, a recent study took advantage of the non-homologous end-joining, which is active throughout the cell cycle, including the G0/G1 phase of non-dividing

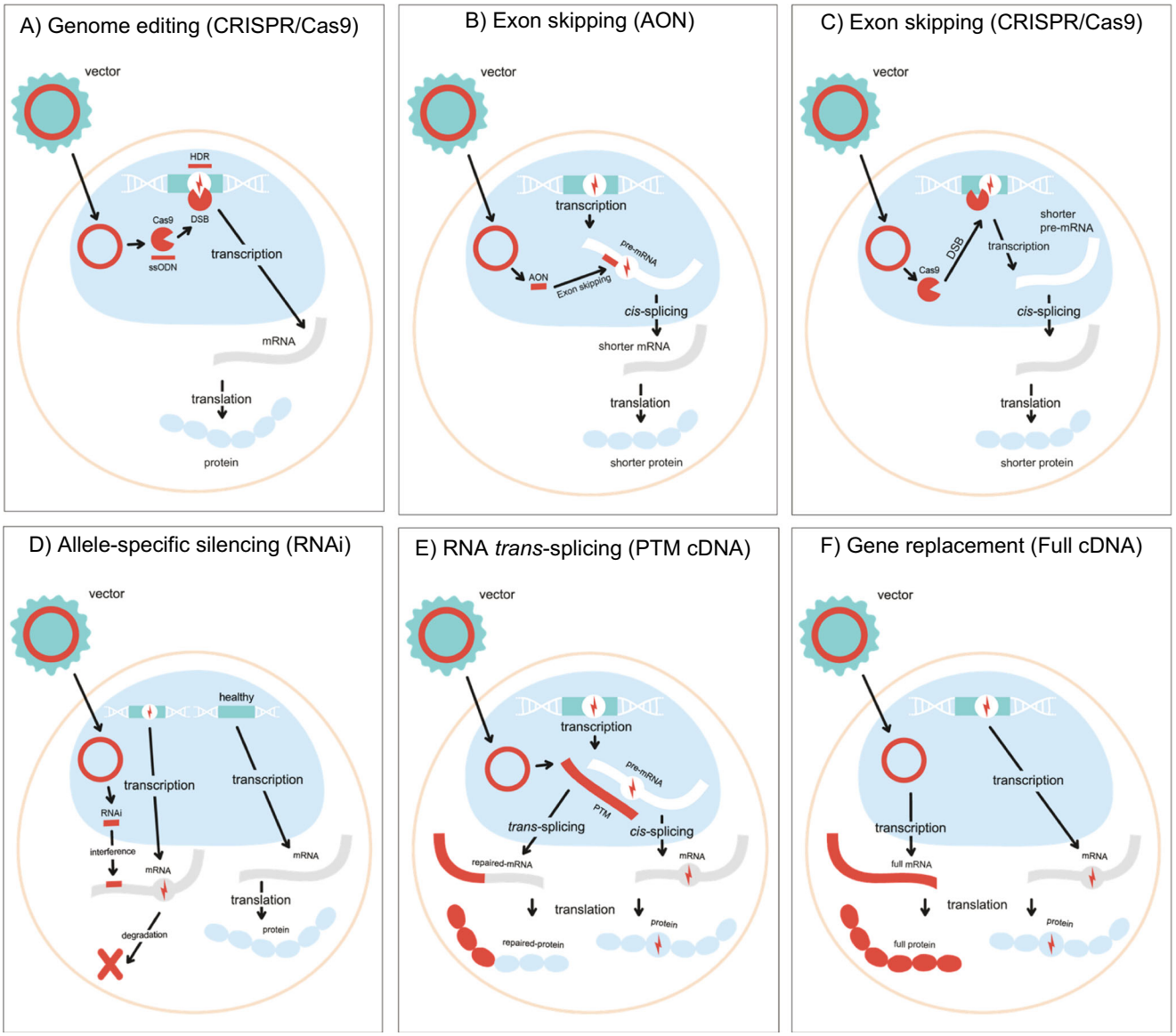

Fig. 1 AAV-mediated gene therapy options for inherited disorders. The depicted gene therapy strategies combine a vector (e.g., adeno-associated virus, AAV) containing the gene therapy medicinal product (red circle) under the control of a promoter, which is delivered to the target cell via the most appropriate administration way such as systemic delivery. Viral particles enter the cells via specific receptors and then the nucleus (light blue), where the gene therapy medicinal product is transcribed to target either the DNA (CRISPR/Cas9), the pre-mRNA (antisense oligonucleotide, pre-*trans*-splicing molecule), the mRNA (RNA interference molecule) or to replace the loss-of-functional endogenous protein (full-length cDNA). **a** Genome editing with CRISPR/Cas9. Cas9 protein is produced within the cell and then targeted to the gene-of-interest with a specific single guide RNA. Cas9 produces double-strand break and the mutation is repaired by homology-directed repair with a repair template. This leads to a functional full-length mRNA and protein. **b** Exon skipping with antisense oligonucleotide. Antisense oligonucleotide targets exonic splicing enhancer sequences of an in-frame mutated exon, preventing binding of proteins involved in the splicing process. This induces the skipping of the mutated exon and the production of a shorter, but functional protein. **c** Exon skipping with CRISPR/Cas9. The specific single guide RNA brings Cas9 towards the targeted DNA sequence to permanently cut in-frame the mutated exon. Consequences are identical as in (**b**). **d** Allele-specific silencing. The mutant mRNA is knocked-down by a specific RNA interference molecule. This leads to its degradation and the absence of mutant protein. **e** RNA *trans*-splicing. The gene therapy medicinal product is a pre-*trans*-splicing molecule, encoding wild-type exonic and intronic sequences, including a specific complementary motif for endogenous target pre-mRNA. The pre-*trans*-splicing molecule hybridizes with the target pre-mRNA to generate a repaired mRNA molecule, devoid of mutation and a full-length functional protein. This process competes with *cis*-splicing, which gives rise to endogenous mRNA and proteins. **f** Gene replacement. The gene therapy medicinal product is a full-length cDNA that will be transcribed and translated into a functional full-length protein that compensates loss-of-function mutations. In the specific case of disease of the sarcomere, it also replaces the transcription and translation of endogenous genes. Abbreviations used are: AON, antisense oligonucleotide; Cas9, CRISPR type-2 system proteins; CRISPR, clustered regularly interspaced short palindromic repeats; DSB, double-strand break; HDR, homology-directed repair; RNAi, RNA interference; PTM, pre-*trans*-splicing molecule; sgRNA, single-guided RNA.

cells, to establish the homology-independent targeted integration for gene targeting [68]. The authors reported an efficiency of ~ 60% after transduction of differentiated neurons and of ~ 3% in heart 2 weeks after AAV9-mediated systemic delivery in neonatal mice.

Moreover, CRISPR/Cas9 is also at the forefront of human germline therapy, since several studies have reported efficient and successful genome editing of human embryos [35, 41, 44, 69]. The study by Ma et al. focused on a male HCM patient with a familial history of HCM caused by a GAGT-deletion in exon 16 of the *MYBPC3* gene [44]. The authors reported that correction of germline mutations was achieved by the activation of an endogenous, germline-specific DNA repair response. Therefore, double-strand breaks at the mutant paternal allele were predominantly repaired using the homologous wild-type maternal gene instead of a synthetic DNA template. Induction of double-strand breaks at specific cell cycle stages was able to increase homology-directed repair efficiencies up to 27% among targeted clones, and led to the prevention of mosaicism. Furthermore, no off-targets events were found. However, these findings remain controversial as inter-homologous recombination was never reported before, and already established methods, such as preimplantation genetic diagnosis, could be used to collect and identify eggs without a mutation for further implantation [9]. This would avoid editing of human embryos and therefore potential off-target effects. Nevertheless, these studies show that treatment of human germline mutations can be accomplished with CRISPR/Cas9.

Exon skipping

The concept of exon skipping strategies is to remove in-frame the exon(s) containing the gene defect(s), thereby creating a shorter, yet functional protein (for review, see [22]). Most of the studies used antisense oligonucleotides (Fig. 1b), which were designed to mask exonic splicing enhancer motifs to prevent binding of the splicing machinery on the endogenous pre-mRNA, ultimately leading to bounced exon(s) and a shorter mature mRNA. In the last years, a great deal of effort has been dedicated to the optimization of the chemistry of antisense oligonucleotides in order to increase their stability and performance. This therapeutic approach has been especially successful in Duchenne muscular dystrophy with the recent approval by the Food and Drug Administration of *eteplirsen*, the first ever antisense oligonucleotide-based drug (for review, see [1]). Besides antisense oligonucleotide, CRISPR/Cas9 genome editing (Fig. 1c) has been recently evaluated to permanently skip in-frame mutated exons of the dystrophin gene in myoblasts [61] or hiPSCs [39, 43] derived from patients with Duchenne muscular dystrophy, as well as in skeletal and cardiac muscles of a new mouse model of Duchenne muscular dystrophy generated with CRISPR/Cas9 [2]. Strikingly, in the latter study, 90% of dystrophin protein level was restored in both skeletal muscle and heart after a single systemic delivery of AAV9 encoding Cas9 and single guide RNAs in neonatal mice.

In the context of HCM, our group evaluated antisense oligonucleotide-based exon skipping as a gene therapy option in *Mybpc3*-targeted knock-in mice [15]. These mice carry a homozygous G>A transition on the last nucleotide of exon 6, which belongs to the highly conserved 5′ splice donor site sequence, resulting in different aberrant mRNAs [72]. This mutation is a founder mutation in Tuscany region in Italy where it accounts for 14% of all HCM cases [19]. In-frame skipping of both exons 5 and 6 of *Mybpc3* with specifically designed antisense oligonucleotides and AAV9-mediated delivery in newborn mice abolished cardiac dysfunction and prevented the development of left ventricular hypertrophy. Although the therapeutic effect was transient, this first proof-of-concept study paves the way towards a causal therapy of HCM [15]. We think that AAV-mediated exon skipping with antisense oligonucleotide(s) or CRISPR/Cas9 remains a valuable therapeutic option for *MYBPC3* mutation hot spots, such as exon 25 containing 11% of all exonic mutations [5].

Allele-specific silencing

Rapid development in allele-specific silencing by RNA interference (Fig. 1d) has proven to be another option to cure autosomal-dominant diseases by targeting the mutant allele in vitro or in vivo in mice [8, 51, 71].

In the context of HCM, this has been used with success to eliminate the mutant allele and delay the progression of cardiomyopathy in *Myh6*-targeted knock-in mice [32]. The limitations of this approach are the same as for exon skipping since several molecules should be designed and generated to target every mutation. On the other hand, this strategy could be of interest to target common single nucleotide polymorphisms, which would be co-transmitted with the mutant allele in patients.

RNA trans-splicing

The goal of this approach is to reduce the level of defective transcripts with a repaired full length mRNA. *Trans*-splicing occurs during mRNA maturation. It is a splicing reaction between two independently transcribed RNA molecules, a target endogenous mutant pre-mRNA and a therapeutic pre-*trans*-splicing molecule (Fig. 1e; for reviews, see [7, 73]). It is an attractive treatment option, since it uses the endogenous spliceosome machinery in the nucleus, does not require delivery of the complete gene and is restricted to those cells expressing the target pre-mRNA. Upon AAV-mediated delivery and transcription in the

nucleus, the pre-*trans*-splicing molecule hybridizes with the target pre-mRNA to generate a chimeric mRNA molecule. Therefore, the amount of repaired mRNA should rise in comparison to the amount of mutant endogenous mRNA. Depending on the position of the mutation in the pre-mRNA, *trans*-splicing can occur in 5′- or 3′-mode and pre-*trans*-splicing molecules will contain a splice donor or acceptor site, respectively. *Trans*-splicing has already proven its potential as a treatment option for genetic diseases such as Duchenne muscular dystrophy and spinal muscular atrophy in vitro and in vivo (for review, see [5].

First evidence for successful 5′ *trans*-splicing in the context of HCM was reported by our group both in cardiomyocytes and *in vivo* in *Mybpc3*-targeted knock-in mice [53]. Although relatively successful in vitro, the repaired *Mybpc3* mRNA represented only 0.14% of the total transcripts in the heart 7 days after AAV9 delivery, which was not enough to prevent the development of cardiac hypertrophy and dysfunction in HCM mice. We recently applied *trans*-splicing in the 5′- and 3′-mode to hiPSC-derived cardiomyocytes from an HCM patient, carrying a truncating *MYBPC3* mutation [63]. Consistently with the report in *Mybpc3*-targeted knock-in mice, we also found very low *trans*-splicing efficiencies and therefore no repaired cMyBP-C protein. Low 5′ *trans*-splicing efficiency was also recently reported in *Lmna*-targeted knock-in mice with maturation defects in skeletal and cardiac muscles [4].

These studies showed that further optimization is needed to increase *trans*-splicing efficiencies, so that it can be taken into consideration as a treatment option in humans. Especially patients with HCM-associated *MYBPC3* mutations could profit from *trans*-splicing because all mutations could be repaired with only two PTM molecules, covering the first and the second half of the *MYBPC3* mRNA [63]. This treatment would therefore benefit 40–60% of all HCM patients (for reviews, see [5, 10]). In addition, *trans*-splicing is expected to result in a repaired full-length and functional cMyBP-C protein, in contrast to exon skipping. Furthermore, the authorization for marketing of two pre-*trans*-splicing molecules as new gene therapy medicinal product would be easier and quicker than for several antisense oligonucleotides, RNA interference or single guide RNA molecules.

Gene replacement

Gene replacement therapy is mainly applied when a mutation results in a low level or absence of the corresponding protein. In this case, full-length wild-type cDNA of the gene-of-interest is packaged and delivered by a vector to the cell of interest (Fig. 1f). Upon successful transduction, the cDNA sequence will find itself in the nucleus and transcription is initiated. The resulting exogenous mRNA is translated into a functional protein that replaces the missing mutant endogenous protein.

Gene replacement is particularly interesting for HCM-associated *MYBPC3* mutations, because they usually result in a low level or absence of mutant proteins, causing haploinsufficiency (for review, see [10]). Additional expression of any sarcomeric protein is expected to replace the endogenous protein level and to prevent haploinsufficiency, because the sarcomere is a tightly regulated system with a preserved protein stoichiometry [70]. We previously showed that delivery of full-length wild-type *Mybpc3* cDNA prevents the development of cardiac hypertrophy and dysfunction by increasing the amount of cMyBP-C protein in *Mybpc3*-targeted knock-in mice [52]. This successful proof-of-concept study in mice was achieved with a single systemic delivery of AAV9 encoding *Mybpc3* under the control of human cardiac troponin T promoter in 1-day-old mice. For the observation period of 34 weeks, *Mybpc3* mRNA and cMyBP-C protein levels increased in an AAV dose-dependent manner. Additionally, *Mybpc3* gene therapy also suppressed accumulation of mutant mRNAs and consequently the production of poison peptides. In agreement to this, we showed that AAV-mediated *MYBPC3* gene transfer dose-dependently restores the force of contraction to wild-type level in engineered heart tissue derived from cardiac cells of neonatal *Mybpc3*-targeted knock-out mice [76]. As an intermediate step towards humans, we further tested this concept in hiPSCs, which were retrieved from an HCM patient carrying a truncating *MYBPC3* mutation in [63]. *MYBPC3* mRNA and cMyBP-C protein levels were ~ 50% lower in patient-specific than in control hiPSC-derived cardiomyocytes. Additionally, patient-specific hiPSC-derived cardiomyocytes exhibited higher cell area and altered gene expression, reproducing human HCM features. AAV-mediated delivery of the human full-length *MYBPC3* cDNA in patient-specific hiPSC-derived cardiomyocytes resulted in higher amounts of *MYBPC3* mRNA and cMyBP-C protein 7 days after transduction. Furthermore, restoration of cMyBP-C haploinsufficiency was sufficient to suppress hypertrophy. Stoichiometry of the sarcomere was also preserved in the unrelated control hiPSC-derived cardiomyocytes, where *MYBPC3* gene transfer resulted in higher levels of mRNA without changes in the level of cMyBP-C protein. Beneficial outcomes using gene replacement therapy were also reported in human embryonic stem cell-derived cardiomyocytes carrying a truncating *MYBPC3* mutation [54]. In this study, adenoviral-mediated gene transfer of full-length *MYBPC3* prevented hypertrophy, sarcomeric disarray and improved calcium impulse propagation in HCM cardiomyocytes 7 days after treatment. Additionally, Western blot analyses suggested more exogenous cMyBP-C protein in HCM than in control cardiomyocytes, which fits to preserved stoichiometry of the sarcomere as described above. No clinical study has been initiated yet using gene replacement therapy for

HCM. Therefore, these studies represent an intermediate step towards clinical application.

Conclusion—future directions

Gene therapy targeting the cause of HCM is particularly attractive for the rare, severe forms with bi-allelic truncating mutations in *MYBPC3* leading to heart failure and premature death in infants [75]. Among the different gene therapy options tested, gene replacement with AAV9-mediated delivery of functional *MYBPC3* cDNA has already proven to be the most successful in mice [52] and in human pluripotent stem cell-derived cardiomyocytes [54, 63]. Gene replacement therapy is attractive for cardiomyopathy of the sarcomere, which is a tightly regulated protein complex with a preserved stoichiometry. Thus, any addition of a functional full-length cDNA should replace, at least in part if not all, the expression of the endogenous protein. Using AAV with a high cardiac tropism and a cardiomyocyte-specific promoter, any kind of sarcomere mutation can theoretically be corrected. Besides gene replacement therapy, AAV9-mediated delivery of Cas9 and single guide RNAs has already proven to be successful to induce in-frame exon skipping and reframing of dystrophin in a mouse model of Duchenne muscular dystrophy [2]. This exciting study certainly paves the way towards first-in-man and may also be a good strategy to address mutation hot spots in sarcomeric genes. However, in all cases, still novel technologies should be tested to augment AAV accumulation in the heart of large animal models and humans such as nanomaterial-coated capsids [36], to further increase the efficiency of CRISPR/Cas9-mediated homology-directed repair or homology-independent targeted integration in the heart in vivo, and to advance knowledge for the best AAV serotype for human heart. Finally, a joint effort between families, (pediatric) cardiologists and cardiac surgeons, gynecologists, geneticists, and basic scientists is necessary to form a basis to advance gene therapy towards first-in-patient, at least for the severe forms of inherited cardiomyopathy.

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Compliance with ethical standard

Conflict of interest L.C. and G.M. are co-applicants and holders of a provisional patent no. PCT/EP2014/057984 (WO 2014/170470 A1; EP2792742 A1; CA2944186 A1), Priority date Apr 17, 2013, Filling date Apr 17, 2014, publication date Oct 23, 2014. The authors declare no competing financial interests.

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