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INVITED REVIEW

MYBPC3 in hypertrophic cardiomyopathy: from mutation identification to RNA-based correction

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Abstract Mutations in *MYBPC3* gene, encoding cardiac myosin-binding protein C (cMyBP-C), frequently cause hypertrophic cardiomyopathy (HCM), which affects 0.2 % of the general population. This myocardial autosomal-dominant disorder is the leading cause of sudden cardiac death particularly in young athletes. The current pharmacological and surgical treatments of HCM focus on symptoms relief, but do not address the cause of the disease. With the development of novel strategies targeting the endogenous mutation, causal HCM therapy is now possible. This review will discuss the current knowledge on HCM from the identification of *MYBPC3* gene mutations to potential RNA-based correction.

Keywords *MYBPC3* mutations · Haploinsufficiency · Poison peptide · Exon skipping · Exon inclusion · Spliceosome-mediated RNA *trans*-splicing

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Introduction

Hypertrophic cardiomyopathy (HCM) is a myocardial disease characterized by left ventricular hypertrophy, mainly involving the interventricular septum, diastolic dysfunction, and myocardial disarray [15]. The prevalence of HCM has been estimated to be 1:500 in the general population [38]. It is therefore the most frequent inherited cardiac disease and the leading cause of sudden cardiac death (SCD), particularly in young athletes [37]. The clinical outcome of HCM varies from a benign asymptomatic course to atrial fibrillation, heart failure, and SCD due to arrhythmias (for detailed reviews, see [14, 21]).

HCM is transmitted in an autosomal-dominant fashion and is caused by more than 1,000 individual mutations in at least 10 genes encoding components of the sarcomere (for detailed recent reviews, see [18, 55]). The *MYBPC3* gene encoding cardiac myosin-binding protein C (cMyBP-C) has been assigned on chromosome 11p11.2 by the group of Labeit [19]. As a component of the sarcomere interacting with myosin, titin, and actin, *MYBPC3* was the ideal candidate gene for the fourth HCM chromosomal locus (*CMH4*), which was identified on chromosome 11, using the segregation analysis of microsatellite markers in a large French family, by the group of Schwartz 20 years ago [7]. By screening for *MYBPC3* mutations in HCM families, the groups of Schwartz and Seidman simultaneously identified the first three disease mutations [5, 63]. Unexpectedly, these mutations disrupted the

reading frame and produced C-terminal truncated cMyBP-C. During the last 2 decades, a body of evidence revealed *MYBPC3* as the most frequently mutated HCM gene, representing about 40–50 % of all HCM mutations, and nonsense or frameshift mutations as the most common in *MYBPC3* [46, 49, 55].

MYBPC3 mutations and molecular mechanisms

The complete structure and sequence of the human *MYBPC3* gene was established in 1997 [6]. It contains more than 21 kbp and is composed of 35 exons of which 34 are coding (Table 1 and Fig. 1a). Exons 10 and 14 are very small (3 bp) and were often not considered, although several pathogenic mutations were found in the flanking introns [16]. Amplifying complementary DNA (cDNA) from human myocardial samples always revealed these small exons.

More than 350 HCM-associated mutations (460 in HGMD professional 2013.2; http://www.hgmd.org/) were identified in *MYBPC3* (Table 2). Although several missense mutations have been described, which should result in stable mutant proteins that could be incorporated into the sarcomere, 64 % of the known *MYBPC3* mutations are frameshift or nonsense (reviewed in [2, 8, 36, 50]). These mutations result in a premature termination codon (PTC) in the transcribed messenger RNA (mRNA) and are expected to produce C-terminal truncated cMyBP-C lacking myosin-binding and/or titin-binding sites [6]. A 25-bp deletion, including the branch point of intron 32, leads to a frameshift due to exon 33 skipping and has been associated with a higher risk of heart failure in 4 % of the population in South Asia [13].

The molecular mechanisms by which *MYBPC3* mutations lead to HCM remain elusive. Findings in humans support the view that cMyBP-C haploinsufficiency is the main molecular mechanism, even for patients with missense mutations [39, 40, 44, 53, 59, 60]. Haploinsufficiency is also involved in cat and mouse models of HCM that carry either a missense or a frameshift mutation [43, 61]. Haploinsufficiency has been shown to result from regulations by the nonsense-mediated mRNA decay and/or the ubiquitin–proteasome system (UPS) in mice [61]. Recent findings in HCM mouse models suggest that adrenergic stress or aging induces UPS impairment and potential accumulation of truncated proteins that may act as poison polypeptides [54, 56]. This suggests that the "poison peptide" mechanism could contribute in worsening the HCM phenotype when the UPS is impaired.

RNA-based therapy

The current therapy of HCM focuses on symptoms relief by pharmacological and/or surgical treatments but does not address the cause of the disease (see detailed reviews [17, 58]). With the development of new strategies that target the endogenous gene or pre-mRNA, it is now possible to envision causal therapy for HCM. RNA-based therapeutic approaches such as exon skipping, exon inclusion, and spliceosome-mediated RNA *trans*-splicing (SMaRT) were developed for neuromuscular disorders during the last decade (see detailed reviews [25, 27, 34]). The development of these approaches for cardiac genetic diseases is very new.

Exon skipping

Exon skipping can be achieved by using antisense oligonucleotides (AONs). AONs are designed to mask exonic splicing enhancer (ESE) motifs and therefore inhibit binding of *trans*-acting regulatory splicing factors that mediate inclusion of specific exons into the mature mRNA [64]. This is expected to result in the skipping of the targeted exons [25, 64]. An important prerequisite for removing one or more exons is that it should be in-frame and result in an internally deleted (shortened) protein with normal or at least near-normal function, in order to rescue the phenotype.

AONs can be delivered as chemically modified molecules or packaged into adeno-associated virus (AAV). A big hurdle of chemically modified AONs is their delivery into the heart [1, 23]. For example, 2′-O-methyl phosphothioates have shown relatively poor exon skipping efficiency in the heart of dystrophin-deficient mice [3, 22, 28]. On the other hand, the phosphorodiamidate morpholino oligomer (PMO) chemistry has a promising potential for the heart [30]. To improve heart delivery, AONs can also be delivered to the spliceosome machinery by using AAV encoding modified U7 or U1 small nuclear RNA (snRNA) and the appropriate AAV serotype for the best cardiotropism [20, 24, 25].

Out of the 34 coding exons of *MYBPC3*, 14 exons (2–4, 8–11, 14, 20, 22, and 24–27) are in-frame and therefore may be skipped to remove the mutations (Fig. 1a). If we discard exons involved in an important protein structure or function (such as phosphorylation or protein binding sites), then the exons 2, 22, 24, 25, 26, and 27 are ideal targets for skipping (Fig. 1b). Finally, skipping of the single exon 25 will remove a total of 34 mutations, which represents about 11 % of exonic *MYBPC3* mutations (Table 2). It is also possible to skip two or more exons at once to keep the reading frame and the function of the protein (Fig. 1b). We recently published the first proof-of-principle study demonstrating exon skipping in a *Mybpc3*-targeted knock-in (KI) mouse model of HCM [20]. The *Mybpc3*-KI mouse carries one of the most frequent human mutations, the G>A transition on the last nucleotide of exon 6 (c.772G>A), which is part of the consensus 5′ splice donor site sequence [61]. This results in three aberrant mRNAs. In addition, we revealed the

Table 1 Exon-intron structure of the human *MYBPC3* gene. The numbering of cDNA and amino acid at the start and end of each exon is indicated

Exon no.	Exon length (bp)	cDNA start	cDNA end	Protein start	Protein end	Intron length (bp)
1	25	c.1A	c.25G	p.Met1	p.Val9	1117
2	267	c.26T	c.292G	p.Val9	p.Glu98	623
3	114	c.293A	c.406.G	p.Glu98	p.Gly136	389
4	99	c.407G	c.505G	p.Gly136	p.Gly169	91
5	149	c.506G	c.654G	p.Gly169	p.Lys218	1232
6	118	c.655G	c.772G	p.Val219	p.Glu258	518
7	49	c.773A	c.821C	p.Glu258	p.Thr274	176
8	30	c.822G	c.851G	p.Thr274	p.Ser284	171
9	54	c.852 T	c.905G	p.Ser284	p.Arg302	396
10	3	c.906A	c.908A	p.Arg302	p.Asp303	382
11	18	c.909C	c.926G	p.Asp303	p.Arg309	256
12	164	c.927G	c.1090G	p.Arg309	p.Ala364	2582
13	133	c.1091C	c.1223G	p.Ala364	p.Ser408	229
14	3	c.1224C	c.1226A	p.Ser408	p.Lys409	114
15	125	c.1227G	c.1351G	p.Lys409	p.Glu451	85
16	106	c.1352A	c.1457G	p.Glu451	p.Trp486	85
17	167	c.1458G	c.1624G	p.Trp486	p.Glu542	421
18	166	c.1625A	c.1790G	p.Glu542	p.Arg597	746
19	107	c.1791G	c.1897G	p.Arg597	p.Glu633	105
20	30	c.1898A	c.1927G	p.Glu633	p.Glu643	1212
21	140	c.1928A	c.2067G	p.Glu643	p.Gln689	246
22	81	c.2068G	c.2148G	p.Gly690	p.Lys716	644
23	160	c.2149C	c.2308G	p.Leu717	p.Asp770	725
24	105	c.2309A	c.2413G	p.Asp770	p.Gly805	110
25	189	c.2414G	c.2602G	p.Gly805	p.Gly868	1379
26	135	c.2603G	c.2737T	p.Gly868	p.Cys913	667
27	168	c.2738G	c.2905C	p.Cys913	p.Gln969	1031
28	89	c.2906A	c.2994G	p.Gln969	p.Gln998	169
29	196	c.2995G	c.3190G	p.Gly999	p.Asp1064	223
30	140	c.3191A	c.3330G	p.Asp1064	p.Met1110	220
31	160	c.3331G	c.3490G	p.Glu1111	p.Gly1164	111
32	137	c.3491G	c.3627G	p.Gly1164	p.Lys1209	307
33	187	c.3628C	c.3814G	p.Pro1210	p.Val1272	190
34	37	c.3815 T	c.3851G	p.Val1272	p.Q1274	128 (stop TGA1275)
35	311	–	–	–	–	

presence of an alternative splice variant deleted of the exons 5 and 6 (Var-4), which was also expressed at a low level in wild-type mice and very stable after gene transfer in cardiac myocytes [20]. To enhance Var-4 expression, we designed two AONs that mask predicted ESEs in exons 5 and 6 (Fig. 2a). AONs were inserted into U7snRNA and packaged in AAV. Transduction of cardiac myocytes or systemic administration of AAV-U7-AON-5–6 increased Var-4 mRNA/protein levels and reduced aberrant mRNAs. Importantly, injection of newborn KI mice abolished cardiac dysfunction and prevented left ventricular hypertrophy [20].

Exon inclusion

Besides exon skipping, the spliceosome could also be forced to include an exon into the mRNA. This process is called exon inclusion and could be used when the mutation itself is expected to result in exon skipping and frameshift. In this case, AONs are directed against exonic splicing silencer (ESS) or intronic splicing silencer (ISS) motifs, which are normally recognized by splicing repressor proteins such as heterogeneous nuclear ribonucleoprotein A1 [29]. Masking the splicing silencers allows exon recognition by the spliceosome and therefore inclusion of the exon into the mRNA. The efficiency

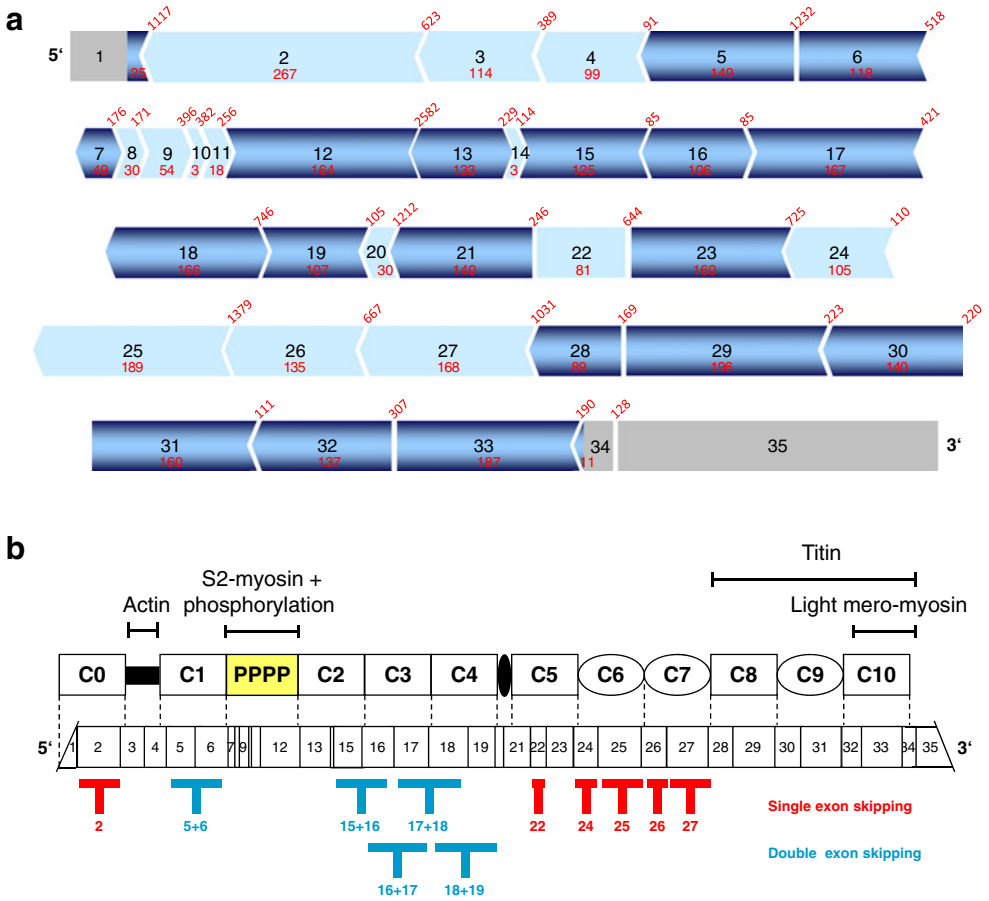

Fig. 1 Structure of human *MYBPC3* exons and cMyBP-C protein domains. **a** Structure of the *MYBPC3* exons. *MYBPC3* gene consists of 35 exons, of which 34 are coding. The untranslated regions (*gray*) and the coding regions (*light and dark blue*) are indicated. Exons in *light blue* could be skipped without inducing a frameshift, whereas exons in *dark blue* could not be skipped without introducing a frameshift. The *red numbers* indicate the sizes (in base pairs) of corresponding exons and introns. Structure was modified from Ensembl. **b** Structure of cMyBP-C protein and *MYBPC3* cDNA. The cMyBP-C protein is composed of 11 domains (C0–C10). Eight are I class immunoglobulin domains (*rectangles*), and three are fibronectin type III domains (*ellipses*). The domains of interaction with other sarcomeric proteins (*solid black lines*) as well as the specific four phosphorylation sites located in the MyBP-C motif (*yellow rectangle*) are indicated. Correspondence between domains and exons is indicated by *dashed lines*. Indicated in the cDNA structure are the exons that could be skipped alone (*red*) and the exons that could be skipped only together to maintain the reading frame (*blue*). Abbreviation used: PPPP, cMyBP-C motif containing the four phosphorylation sites

of exon inclusion can be increased by using bifunctional AONs containing an antisense sequence complementary to the silencer plus a potent enhancer sequence to support the binding of splicing enhancer proteins [9].

Exon inclusion application was used successfully in different spinal muscular atrophy (SMA) mouse models as well as in SMA patient-derived fibroblasts [29, 47, 57]. To date, no studies have been published using exon inclusion to treat HCM. Splicing mutations (and in general, intronic mutations leading to exon skipping) are targets for exon inclusion. Out of the 54 described intronic *MYBPC3* mutations (Table 2), 40 (11 % of the total) are located in splice sites and are therefore candidates for exon inclusion. The limitation of exon inclusion is that a specific AON has to be generated for every single mutation, whereas for exon skipping, a single AON can be used for patients with different mutations in the targeted exon. Since every single AON is considered as a new drug and has to go through all clinical trials individually, exon skipping is more attractive.

Spliceosome-mediated RNA *trans*-splicing

Another approach to target mutant pre-mRNA is the spliceosome-mediated RNA *trans*-splicing (SMaRT) in which two independently transcribed RNA molecules, a target mutant pre-mRNA, and a therapeutic pre-*trans*-splicing molecule (PTM) are spliced together (for review, see [62]). As a result, a full-length repaired mRNA is formed. *Trans*-splicing is useful to treat dominant diseases, and it is a suitable alternative to repair a mutant pre-mRNA when exon skipping and/or exon inclusion is not feasible. *Trans*-splicing is carried out by the endogenous splicing machinery and is restricted to

Table 2 Number of exonic and intronic *MYBPC3* out of a total of 357 mutations

Exon	Missense/small deletion	Frameshift/ nonsense	Polymorphism (silent mutations)	Total exonic mutations	Intronic mutations (splicing/branch point)
1	1	–	1	2	1
2	6	7	2	15	–
3	–	2	–	2	1
4	2	1	4	7	3
5	8	7	4	19	–
6	8	3	–	11	7
7	4	–	1	5	1
8	3	–	–	3	–
9	–	1	–	1	–
10	–	1	–	1	1
11	–	2	–	2	2
12	4	2	1	7	1
13	1	6	2	9	6
14	–	–	–	–	2
15	3	8	–	11	3
16	5	3	–	8	3
17	13	3	5	21	2
18	4	11	–	15	–
19	4	1	2	7	–
20	–	–	–	–	1
21	6	2	1	9	–
22	1	3	–	4	1
23	5	10	–	15	4
24	3	5	–	8	–
25	13	21	–	34	1
26	2	4	1	7	1
27	5	10	–	15	3
28	1	1	–	2	1
29	7	8	1	16	4
30	2	8	1	11	3
31	6	4	–	10	1
32	3	3	–	6	1
33	9	11	–	20	–
34	–	–	–	–	–
Tot	129	148	26	303 (85 %)	54 (15 %)

those cells expressing the target pre-mRNA, minimizing the risk of ectopic expression of the repaired mRNA. This approach allows the replacement of endogenous mutations at pre-mRNA level with wild-type coding sequences enclosed in the PTMs. In addition, PTMs should also carry conserved splicing sequences together with a binding domain complementary to the target intronic region. The binding domain is crucial for the specificity of the PTM, and it is assumed that its length positively correlates with its specificity. The feasibility of *trans*-splicing in vitro has been shown in several studies, most of them using the 3′-*trans*-splicing approach [45, 51, 52]. Replacement of an internally mutated exon is the most elegant *trans*-splicing procedure, and its feasibility to repair Duchenne dystrophin transcripts in vitro was recently shown [35]. In the last decade, the therapeutic potentiality of *trans*-splicing was successfully applied to correct in vivo genetic diseases such as SMA [11, 12], tauopathies [4], and hemophilia A [10], all of them being 3′-*trans*-splicing approaches. Moreover, in a mouse model of SMA, it was demonstrated that the combination of *trans*-splicing and AONs to reprogram the mutant SMN2 pre-mRNA is able to lessen the severity of the SMA phenotype, therefore extending the survival of the mice [11].

Recently, our group published the first in vitro and in vivo applications of 5′-*trans*-splicing for HCM (Fig. 2b; [41]). In

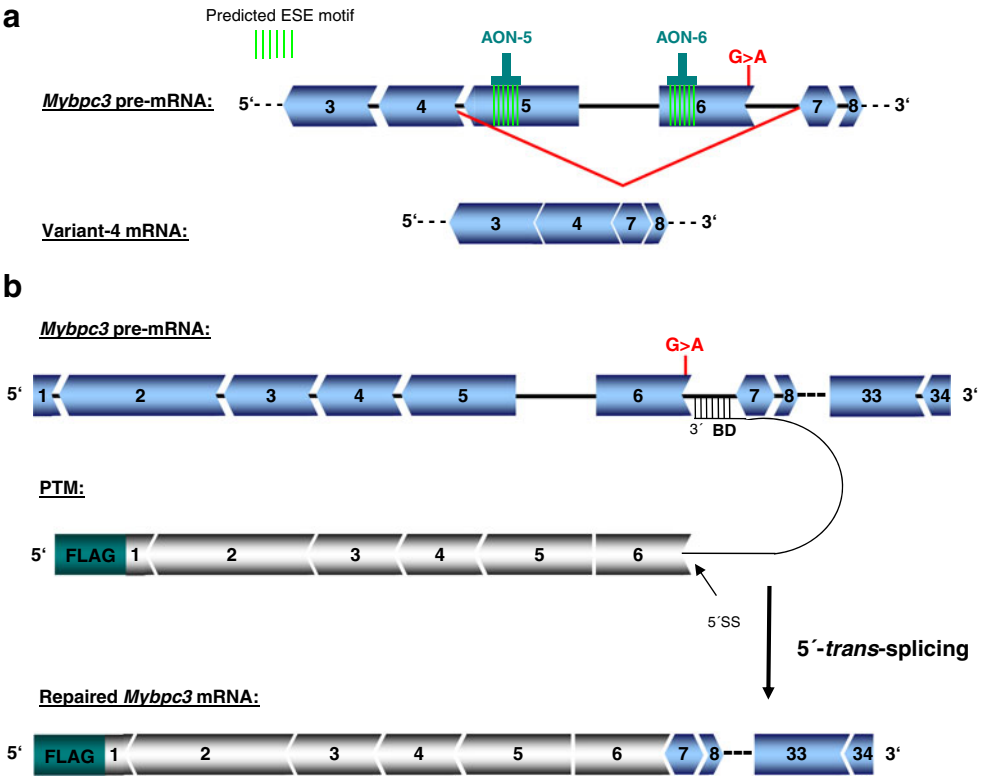

Fig. 2 RNA-based approaches to remove a G>A transition in the last nucleotide of exon 6 of mouse *MYBPC3*. **a** Exon skipping strategy to remove the mutated exon 6 together with exon 5 to maintain the reading frame in *MYBPC3* mRNA using antisense oligonucleotides (AONs) complementary to exonic splicing enhancer (ESE) motifs (*green stripes*) in exon 5 (*AON-5*) and exon 6 (*AON-6*). **b** Schematic representation of 5′-*trans*-splicing bypassing the G>A transition. A pre-*trans*-splicing molecule (PTM), containing the 5′-splicing site (5′SS) and a specific binding domain (BD) complementary to intron 6, was generated to interfere with the endogenous cis-splicing and to produce full-length repaired *MYBPC3* mRNA. Figure adapted from [20, 41]

this study, the feasibility and efficacy of 5′-*trans*-splicing were tested in neonatal cardiac myocytes and in the heart of *Mybpc3*-targeted KI mice. The therapeutic PTM was designed to have a specific cardiac myocyte promoter (human *TNNT2* promoter), and the SV40 polyadenylation (polyA) signal was deleted to maintain the PTM transcripts in the nucleus and to prevent its translation. The packaging in cardiac-specific AAV serotypes allowed efficient transduction of cardiac myocytes ex vivo or in the heart in vivo. However, the translation into a corrected cMyBP-C protein was poor. Even though the efficiency of this process needs to be optimized, it is conceivable to generate two PTM molecules, targeting mutations located in either the 5′ or 3′ regions of *MYBPC3* pre-mRNA in order to treat all HCM-associated *MYBPC3* mutations.

Conventional gene therapy

In larger animal models, conventional cardiac gene therapy has been tested for heart failure (HF), a nongenetic cardiac disease, targeting proteins involved in calcium handling such as phospholamban [32] and S100A1 [48]. Recently, the successful completion of phase II trials for gene therapy of SERCA2a demonstrates the feasibility and safety of AAV1-mediated gene transfer and improvement of the symptoms, and exercise capacity of the patients with advanced HF [31].

Haploinsufficiency of cMyBP-C likely plays a major role in HCM pathogenesis [39, 40, 55]. Insufficient amount of cMyBP-C could produce an imbalance in the stoichiometry of the thick filaments and alter sarcomeric structure and function. Therefore, conventional gene therapy could be also used to treat or prevent the disease. One should note, however, that the presence of poison peptide could hinder the presumed positive effect of *MYBPC3* gene transfer. Spontaneous larger animal models for genetic diseases are rare; however, a cat model carrying an *MYBPC3* mutation associated with cardiac hypertrophy has been described [43]. Last year, it has been shown that direct myocardial injection of a lentivirus-encoding mouse *Mybpc3* cDNA improved myofilament contractile functions in *Mybpc3*-targeted deficient mice [42]. However, the use of lentivirus and the arduous delivery method in this study limit the direct application of this method to HCM patients.

Conclusion and future directions

MYBPC3 gene mutations are the most frequent cause in HCM and are associated with variable clinical manifestations. There is a need for novel therapies, which will be curative, particularly for the severe forms of the disease, leading to a very bad prognosis. The cat model of HCM, which carries a missense *MYBPC3* mutation leading to both haploinsufficiency and

poison peptide, represents a good model to evaluate different gene-based therapeutic strategies. Furthermore, developing new models through HCM human-induced pluripotent stem cells (hIPSC; [33]), such as cardiac myocytes or engineered heart tissue [26], should enable high-throughput investigation of molecular mechanisms and molecular therapy for *MYBPC3*-related HCM. Both the cat and hIPSC-derived cardiac myocytes and engineered heart tissue are required steps before development of clinical trials.

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