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Rescue of cardiomyopathy through U7snRNA-mediated exon skipping in Mybpc3-targeted knock-in mice

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Exon skipping mediated by antisense oligoribonucleotides (AON) is a promising therapeutic approach for genetic disorders, but has not yet been evaluated for cardiac diseases. We investigated the feasibility and efficacy of viral-mediated AON transfer in a Mybpc3-targeted knock-in (KI) mouse model of hypertrophic cardiomyopathy (HCM). KI mice carry a homozygous G>A transition in exon 6, which results in three different aberrant mRNAs. We identified an alternative variant (Var-4) deleted of exons 5–6 in wild-type and KI mice. To enhance its expression and suppress aberrant mRNAs we designed AON-5 and AON-6 that mask splicing enhancer motifs in exons 5 and 6. AONs were inserted into modified U7 small nuclear RNA and packaged in adeno-associated virus (AAV-U7-AON-5+6). Transduction of cardiac myocytes or systemic administration of AAV-U7-AON-5+6 increased Var-4 mRNA/protein levels and reduced aberrant mRNAs. Injection of newborn KI mice abolished cardiac dysfunction and prevented left ventricular hypertrophy. Although the therapeutic effect was transient and therefore requires optimization to be maintained over an extended period, this proof-of-concept study paves the way towards a causal therapy of HCM.

INTRODUCTION

RNA-based therapeutics and splice-switching approaches have been developed over the last decade to treat cancer, asthma, rheumatoid arthritis or neuromuscular diseases such as Duchenne muscular dystrophy (DMD), spinal muscular atrophy and myotonic dystrophy type 1 (for reviews, see Aartsma-Rus et al, 2007; Aartsma-Rus and van Ommen, 2007; Le Roy et al, 2009; Lu et al, 2011). Several studies have shown successful restoration of the DMD reading frame by exon skipping using 2′-O-methyl phosphorothioate-(2OMePS)-antisense oligoribonucleotides (AONs) in mouse (mdx); Bremmer-Bout et al, 2004; Lu et al, 2003) and dog (golden retriever muscular dystrophy, GRMD; McClure et al, 2006) disease models as well as in patient-derived muscle cell cultures (Aartsma-Rus et al, 2007; Madden et al, 2009; van Deutekom et al, 2001, 2007), and recently by systemic administration in DMD patients (Goemans et al, 2011). However, although the exon skipping strategy using back-bone modified AONs has been extensively studied and is already validated in clinical trials, it shows limitations, such as the need for short-term repeated injections and lack of efficient exon skipping in the heart. This limitation may be circumvented by using viral-mediated gene transfer such as adeno-associated viral (AAV) vectors. Tail-vein administration of AAV encoding a...
modified U1 or U7 small nuclear RNA (snRNA) carrying antisense sequences that induce skipping of exon 23 restored and maintained dystrophin expression in cardiac and skeletal muscle of mdx mice until 74 weeks (Denti et al, 2008; Goyenvalle et al, 2012a, 2004). Similarly, percutaneous transcatheter delivery of AAV encoding U7snRNA carrying AON sequences directed against exons 6 plus 8 reduced delivery of AAV encoding U7snRNA carrying AON sequences directed against exons 6 plus 8 reduced.

**RESULTS**

**Evidence for a naturally spliced Mybpc3 mRNA variant in knock-in and wild-type mice**

Mybpc3-targeted KI mice carry a C->A transition on the last nucleotide of exon 6 (Vignier et al, 2009), which is associated with a severe phenotype and bad prognosis in humans (Richard et al, 2003). This mutation was found in ~13% of all HCM patients and in 30% of all MYBPC3 mutation carriers in a large HCM cohort in Italy (Olivotto et al, 2008). Analysis of 10-week-old KI mice revealed higher myofilament Ca²⁺ sensitivity, diastolic and systolic dysfunction and LVH (Fraysse et al, 2012). Previous analysis of KI ventricular tissue indicated normal level of pre-mRNA, but markedly reduced levels of Mybpc3 mutant mRNAs and proteins (Vignier et al, 2009). RT-PCR analysis of KI NMCMs with Mybpc3-specific primers revealed 4 different fragments (Fig 1A). Three bands were previously described and correspond to mutants 1-3 (Vignier et al, 2009). Mutant 1 (Mut-1, missense mRNA) contains the C->A transition and is expected to produce a full-length 150-kDa E264K mutant protein. Mutant 2 (Mut-2, nonsense mRNA) is deleted of exon 6 and expected to result in a premature termination codon (PTC) in exon 9, resulting in a 32-kDa mutant protein. Mutant 3 (Mut-3) is deleted of exon 6, but retains part of intron 8 that restores the reading frame and produces a 147-kDa mutant protein. The newly detected band of 148 bp (variant-4; Var-4) corresponds to a mRNA that is in-frame deleted of exons 5 plus 6 and expected to result in a 139-kDa protein (Fig 1A and B).

To investigate whether Var-4 is a naturally occurring alternative mRNA isoform, RNA from wild-type (WT) NMCMs was used for two rounds of PCR amplification with primers complementary to exons 4 and 7 (Fig 2A). After the first round of PCR the expected 406-bp fragment was obtained in WT. The second round of PCR revealed an additional 139-bp fragment, which corresponded to the fusion of exon 4 with exon 7 (Supporting Information Fig S1), suggesting that Var-4 is an alternative spliced isoform present at low level in WT mice. Var-4 mRNA was detected in NMCMs isolated from WT mice (C57BL/6J) and in ventricular tissue of WT and KI mice (either C57BL/6J or Black swiss) during the entire development (Supporting Information Fig S2). To further evaluate the stability and phosphorylation of Var-4 protein, HEK293 cells were transiently transfected with plasmids encoding FLAG-tagged WT, Mut-1, Mut-2, Mut-3 and Var-4 cMyBP-Cs. Var-4 protein level did not differ from WT, Mut-1 and Mut-3, suggesting that it is very stable. On the other hand, Mut-2, expected at 32kDa, was not detected, supporting previous findings of its rapid degradation by the UPS (Fig 2B; Sarikas et al, 2005). We then investigated whether Var-4 is phosphorylated by PKA. Transfected HEK293 cells were cultured in the presence or absence of forskolin and 3-isobutyl-1-methylxanthine, and cMyBP-C phosphorylation was evaluated using antibodies.
directed against Ser-273, Ser-282 and Ser-302 residues. The basal level of phosphorylated cMyBP-C did not differ between Var-4, Mut-1 and WT (Fig 2C). Phosphorylation of Mut-3 was only detected at Ser-302, as expected from the frameshift (Fig 2C). Activation of PKA increased the phosphorylated cMyBP-C level at Ser-273, Ser-282, and to a lower extent at Ser-302 in Var-4, Mut-1 and WT, and only at Ser-302 in Mut-3 (Fig 2C). Finally, we investigated whether Var-4 protein is incorporated into the sarcomere. KI NMCMs were transduced with an AAV serotype 6 (AAV6) encoding the FLAG-tagged Var-4 protein. Analysis was performed by confocal microscopy using antibodies directed against cMyBP-C, titin and the FLAG epitope (Fig 2D and E). FLAG-Var-4 protein exhibited a striated pattern, was co-localized with cMyBP-C, but not with titin. This suggests that Var-4 protein is well incorporated into the A-band of the sarcomere.

Together, these data suggest that Var-4 protein is likely functional and not toxic. We therefore hypothesized that enhancing its expression would increase the level of cMyBP-C in the sarcomere and therefore ameliorate cardiac function.

Antisense oligoribonucleotides efficiently induce exon skipping in cardiac myocytes

To enhance the expression of Var-4, we designed AONs that mask exonic splicing enhancer (ESE) motifs in exon 5 (AON-5) and in exon 6 (AON-6) of Mybpc3, and are therefore expected to induce an in-frame exon skipping (Fig 3A). Since the mutation also results in the skipping of exon 6 (Mut-2 and Mut-3; Fig 1A

![Figure 1. Molecular impact of the mutation in Mybpc3-targeted KI mice.](image-url)
Figure 2.
and B), we assumed that AON-5 or AON-5 plus AON-6 (AON-5+6), but not AON-6 alone would induce skipping of both exons in KI NMCMs. The specificity of the designed AONs was evaluated in WT and KI NMCMs using AON-5 or AON-5+6. Each AON was full-length modified by 20MePS groups to confer nuclease resistance. Analysis was performed by RT-PCR 8 days after transfection. Whereas treatment of WT NMCMs with AON-5 resulted in the skipping of exon 5 only, treated KI NMCMs presented the skipping of both exons. Treatment with AON-5+6 together resulted in skipping of both exons in WT and KI NMCMs (Supporting Information Fig S3A and S3B). In KI NMCMs exon skipping was accompanied by reduced amounts of mutant mRNAs. Skipping of exons 5 and 6 resulted in the accumulation of Var-4 protein by Western blot (Supporting Information Fig S3C). These results indicate that the exon skipping strategy works in NMCMs and results in specific skipping of the targeted exons.

For further experiments, we produced AAV encoding modified U7snRNA (Goyenvalle et al, 2004) carrying either AON-5 alone or AON-5+6 sequences (Fig 3B). U7 is a non-spliceosomal snRNA, which is normally involved in histone pre-mRNA 3'-end processing (Galli et al, 1983). By a small change in the sequence for the binding site of Sm/Lsm proteins (SmOPT; Stefanovic et al, 1995), it can be redirected to the spliceosome and used as a shuttle for antisense sequences. Here, the sequence was embedded into a snRNPs particle and thus protected from degradation. WT and KI NMCMs were transduced with AAV6 encoding GFP or U7-AON-5+6 at a MOI of 10,000 or were not-transduced (NT). The proportion of Var-4 mRNA was 5–6-fold higher in KI NMCMs transduced with U7-AON-5 or with U7-AON-5+6 than in NT or GFP-transduced KI NMCMs (Fig 4A). This was accompanied by marked reduction or disappearance of Mut-1 mRNA. On the other hand, whereas U7-AON-5 or U7-AON-5+6 did not induce exon skipping in WT NMCMs 48 h after transduction (Fig 4A), it did so 72 h after transduction, but the extent was much less than in KI (Supporting Information Fig S4). Nonsense Mybpc3 mRNA deleted of either exon 5 or exon 6 was stabilized by the translational inhibitor emetine, indicating that they are normally degraded by the NMD in WT cells (Supporting Information Fig S4). We next investigated the efficiency of exon skipping at the protein level by Western blot (Fig 4B). As expected from the mRNA analysis, no Var-4 protein was detected in WT NMCM transduced with U7-AON-5 or U7-AON-5+6 48 h after transduction. On the other hand, the proportion of Var-4 protein was higher in KI NMCM transduced with U7-AON-5 or U7-AON-5+6 than in NT KI NMCMs or those transduced with GFP (Fig 4B).

**AAV9-U7-AON-5+6 induces efficient exon skipping in the heart of 4-week-old KI mice**

To evaluate the efficiency of exon skipping in the mouse heart *in vivo*, we used AAV serotype 9 (AAV9), which has been shown to have a high cardiotoxicity in mice (Inagaki et al, 2006). Phosphate buffered saline (PBS), sodium chloride (NaCl), or AAV9 encoding GFP or U7-AON-5+6 were administered in 4-week-old KI mice by tail-vein injection. As a further control, one WT mouse received NaCl. The expression of GFP was evaluated by RT-PCR in different tissues (heart, lung, and kidney) 3–5 weeks after injection. GFP mRNA level was markedly higher in the heart than liver, lung and kidney (Supporting Information Fig S5), confirming the cardiotoxicity of AAV9. Systemic administration of U7-AON-5+6 (9.4 \( \times \) 10^{11} viral genome (vg)/mouse, which corresponds to a mean dose of 4.8 \( \times \) 10^{13} vg/kg of body weight (BW)) markedly induced the skipping of exons 5 plus 6 and resulted in a higher level of Var-4 mRNA than in control mice 4 weeks after (\( \sim \) 55% of total vs. a mean of 25% in controls; Fig 5A). This was accompanied by reduced amounts of Mut-1 mRNA. To detect Var-4 protein we used two different antibodies, one directed against the N-terminal domain of cMyBP-C (cMyBP-C antibody) and the second one directed against the amino acids produced by the fusion of exon 4 with exon 7 (Var-4 antibody). Both revealed that U7-AON-5+6 increased the Var-4 protein level when compared to PBS or GFP-injected KI mice (Fig 5B).

Cardiac function was evaluated by echocardiography in WT mice that had received PBS and in KI mice that had received PBS or AAV9 encoding U7-AON-5+6 4 weeks before. Compared to WT animals, KI mice treated with PBS exhibited lower fractional area shortening (FAS) and higher left ventricular mass-to-BW ratio (LVM/BW; Fig 5C). The cardiac phenotype was not improved after U7-AON5+6 administration. This suggests that (i) LVH and cardiac dysfunction cannot be rescued in KI mice at this stage or (ii) that the dose of virus was too low.

**AAV9-U7-AON-5+6-mediated exon skipping restores cardiac function and prevents left ventricular hypertrophy in newborn KI mice**

To investigate these hypotheses further, we used newborn mice. Echocardiography was first performed in untreated...
1-d-old WT and KI mice (Fig 6A). KI mice exhibited higher left ventricular internal diameter (LVID) both in diastole and in systole, which resulted in lower FAS than in WT mice. LVM did not differ between the groups, but BW was lower in KI than in WT mice. Therefore the resulting higher LVM/BW was not the result of LVH in KI mice. These data indicate that 1-d-old KI mice exhibit only LV dilation and dysfunction without LVH. Analysis of the foetal gene program revealed higher mRNA levels for atrial natriuretic peptide (Nppa) and brain natriuretic peptide (Nppb) in 1-d-old KI than WT mice, whereas \( \text{b-myosin heavy chain (Myh7)} \) and \( \alpha\)-skeletal actin (Acta1) mRNA levels did not differ among the groups (Fig 6B). In order to determine when LVH started in KI mice, we evaluated KI and WT mice at postnatal days 1, 2, 4 and 7 (Fig 6C). Heart weight (HW) and therefore HW-to-body weight ratio (HW/BW) started to be higher at day 4 and were both markedly higher at day 7 in KI than in WT mice. This was paralleled by the accumulation of \( \text{Acta1 mRNA in KI at day 4. Interestingly, Myh7 mRNA level remained high in KI whereas it decreased quickly after birth in WT mice.} \)

Then, we evaluated whether U7-AON-5+6 prevents LVH and rescues cardiac function in neonatal KI mice. AAV9 encoding U7-AON-5+6 was administered into the temporal vein (Dominguez et al, 2011) of 1-2-day-old KI mice and results were compared to KI and WT mice that received PBS. Each mouse received \( 2 \times 10^{11} \) vg of AAV9 corresponding to a mean dose of \( 1.44 \times 10^{14} \) vg/kg BW. This relative dose was threefold higher than the dose administered in 4-week-old mice. Evaluation of GFP expression by immunofluorescence in cardiac sections suggested high transduction efficiency 7 days after injection (Supporting Information Fig S5). Echocardiography was performed 7 days after U7-AON-5+6 administration (Fig 7). As expected, LVM/BW was higher and FAS lower in PBS-treated KI than in WT mice. Strikingly, both...
parameters were completely rescued in KI mice that received U7-AON-5+6, and therefore did not differ from WT parameters. To evaluate whether the functional improvement was associated with exon skipping, another series of 1-d-old KI mice was treated with U7-AON-5+6 or PBS for 7 days, and molecular analyses were performed in ventricular tissue (Fig 8). RT-PCR showed higher Var-4 and Mut-2 mRNA levels, lower levels of Mut-1 + Mut-3 mRNA, and higher levels of total Mybpc3 mRNA in the U7-AON-5+6- than PBS-treated group (Fig 8A). Western blot analysis with a cMyBP-C antibody directed against the MyBP-C motif performed on Urea protein fractions revealed two bands in both groups, corresponding to Mut-1/-3 and to Var-4, respectively. A shift was observed towards the Var-4 isoform and higher level of total cMyBP-C in most of the mice that received U7-AON-5+6 (Fig 8B). Immunofluorescence analysis of mouse heart sections did not show major differences in the sarcomeric pattern between PBS- and U7-AON-5+6-treated mice (Supporting Information Fig S6).

To assess whether the higher level of Var-4 could be the cause of the improved cardiac phenotype, we evaluated the consequence of systemic delivery of AAV9 encoding FLAG-Var-4 in 1-d-old KI mice 7 days after administration (Supporting Information Fig S7). FLAG-Var-4 mRNA and protein were detected in the heart 7 days after AAV9 administration. Echocardiographic analysis revealed lower LVM/BW and higher FAS in Var-4- than PBS-treated KI mice. This was accompanied by the complete inactivation of the expression of the foetal genes, except for Myh7. This indicates that Var-4 functionally substitutes for WT cMyBP-C.

We finally evaluated whether the rescue of the functional and molecular phenotype persisted over a longer period of time (Fig 9). In AAV9-treated mice, LVM/BW remained stable and lower than in PBS-treated KI mice over the observation period of 55 days after AAV9-U7-AON-5+6 injection, but FAS decreased (Fig 9A). This pattern reflected that of WT mice at a slightly lower level, but clearly differed from PBS-treated KI mice, which started with low FAS that rather increased with time. On the other hand, the skipping of exons 5 plus 6 was persistent and
resulted in a 2-fold higher level of Var-4 mRNA than in PBS-treated KI mice (Fig 9B). This was accompanied by reduced amounts of Mut-1 mRNA. Var-4 protein was also stabilized 55 days after treatment (Fig 9C). In order to understand why the rescue was not fully maintained over time, we analysed viral particle density in the heart (Supporting Information Fig S8). Virus genome was detected only in mice, which received AAV9 and the quantity was 4-fold lower 55 days than 7 days after administration. This may suggest the need for higher virus doses to maintain functional rescue.

**Figure 5. Impact of AAV9-U7-AONs on molecular and functional phenotype in 4-week-old mice.** KI mice received NaCl, PBS, adeno-associated virus serotype 9 (AAV9) encoding GFP (GFP, 7.6 × 10^{10} vg) or encoding U7-AON-5+6 (9.4 × 10^{11} vg) by systemic administration into the tail vein. Analyses of ventricular tissue were performed 4 weeks post-injection.

A. RT-PCR analysis using primers located in exons 4 and 9 of Mybpc3 and α-cardiac muscle actin (Actc1). The level of mutant Mybpc3 mRNAs was quantified using the Gene Tool Software (Syngene, Cambridge) and the level of Var-4 mRNA was expressed as percentage of total Mybpc3 mRNAs, and indicated in the figure.

B. Western blot stained with antibodies directed against the N-terminus of cMyBP-C (cMyBP-C), the amino acids produced by the fusion of exon 4 with exon 7 (Var-4) or against α-actinin. The Var-4-cMyBP-C antibody also detects WT, Mut-1 and/or Mut-3 cMyBP-C proteins. As positive controls, protein extracts from either ventricular tissue of a wild-type mouse injected with PBS (WT-NaCl) or from HEK293 cells transfected with a plasmid encoding Var-4 were used (HEK-Var-4). The expected fragments are indicated by arrowheads.

C. Echocardiographic analyses were performed 4 weeks after administration of AAV9 or PBS in KI and/or WT mice. Fractional area shortening (FAS), left ventricular mass-to-body-weight (LVM/BW) ratio are shown. Data are expressed as mean ± SEM. ***p < 0.001 and **p < 0.01 versus PBS-treated WT, one-way ANOVA and Bonferroni post-hoc test. Number of animals is indicated in the bars. M, 100-bp molecular weight marker.
Figure 6. Evaluation of the cardiac and molecular phenotype in neonatal mice.

A. Echocardiographic analyses were performed in 1-d-old WT and KI mice. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001 versus WT, Student’s t-test (n = 7).

B. RT-qPCR of hypertrophic markers in ventricular tissue of 1-day-old KI and WT mice. Data are expressed as mean ± SEM, ***p < 0.001 versus WT, Student’s t-test (n = 7).

C. Evaluation of the cardiac phenotype and RT-qPCR of hypertrophic markers in ventricular tissue of 1-7-day-old KI and WT neonatal mice. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 versus WT, same age, and *p < 0.05 and **p < 0.01 versus 1-day-old WT, two-way ANOVA Bonferroni post hoc test (n = 4). Gray and white bars correspond to KI and WT mice, respectively. Acta1, α-skeletal actin; BW, body weight; FAS, fractional area shortening; HW, heart weight; LVIDd, left ventricular internal diameter in diastole; LVIDs, left ventricular internal diameter in systole; LVM, left ventricular mass; Nppa, atrial natriuretic peptide; Nppb, brain natriuretic peptide; Myh7, β-myosin-heavy chain.
DISCUSSION

This is the first proof-of-principle study demonstrating that AONs induce exon skipping and the production of in-frame modified mRNA and protein in isolated cardiac myocytes and in the heart in vivo in a Mybpc3-targeted KI mouse model of HCM. The major findings of the present study are: (i) a new alternative spliced isoform of Mybpc3 deleted of exons 5 and 6 (Var-4) was identified in both WT and KI mice; (ii) gene transfer of Var-4 in HEK293 cells and in NCMs resulted in a stable protein, phosphorylated by PKA, and correctly incorporated into the sarcomere, (iii) Var-4 expression was increased using AONs directed against ESE motifs located in exons 5 and 6 of Mybpc3 in cardiac myocytes; (iv) systemic administration of AAV9 encoding modified U7snRNA carrying AON-5+6 induced accumulation of Var-4 mRNA and protein in KI mice; (v) importantly, systemic delivery of AAV9 encoding U7-AON-5+6 abolished systolic dysfunction and prevented development of LVH in neonatal KI mice. Although the therapeutic effect was transient, this study represents the first step towards molecular-based therapy of HCM.

Although there are some reports of exon skipping in the heart of animal models of DMD, this strategy was never evaluated in the context of HCM, and more generally in the context of cardiac genetic diseases. In our study, analyses were performed in homozygous KI mice, which bear a Mybpc3 point mutation that is located in the consensus splice donor site sequence of exon 6. This mutation was found in ~13% of HCM patients and represents therefore the most frequent HCM mutation, at least in Italy (Olivotto et al., 2008). Characterization of KI mice revealed three mutant mRNAs (Vignier et al., 2009). In the present study, we identified a new mRNA isoform lacking exons 5 and 6 in NCMs and ventricular tissue of KI and WT mice of different genetic backgrounds (Figs 1 and 2, and Figs S1 and S2). This suggests the existence of a naturally spliced Mybpc3 mRNA isoform, which was not described in the literature and that we name Var-4. Our data indicate stability, normal phosphorylation and incorporation of Var-4 protein into the sarcomere (Fig 2).

Var-4 could thus be a “therapeutic molecule” that keeps its regulating properties during contraction and relaxation. We hypothesized that strategies aiming at increasing its level could rescue the molecular and functional phenotype.

The only study using 2OMePS-AONs in NCMs was described recently (Wang et al., 2010). The authors established a cardiac myocyte model to screen AONs for skipping of the mutated exon 23 in mdx mice, a model of DMD, and revealed low skipping efficiency (~5%). Other studies investigated systemic delivery of 2OMePS-AONs in mdx mice and revealed a very low or rather non-existent exon skipping efficiency in the heart (Heemskerk et al., 2009; Lu et al., 2005). Direct intra-cardiac injection of naked morpholino AONs resulted in local exon skipping and dystrophin production, but at much lower levels than observed after intramuscular injection in skeletal muscles of mdx mouse (Vitiello et al., 2008). This observation was likely due to the fact that skeletal muscle fibers of DMD mutation-carriers show damages and membrane tears and are thus more diffusible than heart cells, which are less affected. In the present study, 2OMePS-AONs were used to test the targeting specificity of the designed AON sequences. We show that transfection of cardiac myocytes with 2OMePS-AON-5 or -AON-5+6 both induced skipping of the corresponding exons from WT Mybpc3 mRNA (Fig S2). Interestingly, KI NCMs treated either with AON-5 or AON-5+6 increased the production of Var-4 mRNA, and this was associated with reduced amounts of mutant 1–3 mRNAs. We suggest that the mutation on the consensus splice donor site of exon 6 destroys an ESE motif, which results in a weak splicing signal followed by the skipping of exon 6 together with the targeted exon 5.

Transduction of KI NCMs with AAV6 encoding U7-AON-5 or U7-AON-5+6 markedly increased the amount of Var-4 mRNA (Fig 4). This completely prevented the formation of Mut-1 mRNA, whereas Mut-2 and Mut-3 mRNA remained. We suggest that besides the skipping of both exons, U7-AON-5+6 also leads to the skipping of individual exon, and thus skipping of only exon 6 would result in Mut-2 and/or Mut-3 mRNAs. This is further supported by the detection of mRNA deleted of exon 5, exon 6 or both exons 5 + 6 after administration of 2OMePS-U7-AON-5+6 or AAV6-U7-AON-5+6 in WT cells (Figs S3 and S4) and by the detection of higher levels of both Var-4 and Mut-2 mRNAs after AAV9-U7-AON-5+6 injection in vivo (Figs 8 and 9). Previous data obtained in myotubes of DMD patients also showed that AONs directed against two different exons result in the skipping of both exons as well as the skipping of the single exons (Aartsma-Rus et al., 2004). On the other hand, this finding may be explained by a potential competition of targeting exon 5 and/or exon 6, which results in the skipping of either both exons, exon 5 or exon 6 in some cells. The single skipping of exon 5 is expected to result in a frameshift and a PTC in exon 6. This
mRNA was unstable and degraded by the NMD (Fig S3). Interestingly, transduction of WT NMCM with AAV6-U7-AON-5+6 did not result in Var-4 mRNA or protein after 48 h and only in low levels after 72 h (Fig S4). This suggests that double skipping is less effective in WT cells and that single exon-skipped mRNAs are quickly degraded by the NMD (Goyenvalle et al, 2012b). This observation may be advantageous for treating dominant diseases by exon skipping.

In vivo gene transfer of AAV9-U7-AON-5+6 efficiently induced the skipping of exons 5 and 6 of Mybpc3 and therefore
Figure 9. Long-term effect of AAV9-U7-AON-5+6 on the functional and molecular phenotype. 1-2-day-old KI mice received PBS or adeno-associated virus serotype 9 (AAV9) encoding U7-AON-5+6 (2 × 10¹¹ vg) by systemic administration into the temporal vein.

A. Echocardiographic analyses were performed at different postnatal windows from 7 days to 55 days post-injection (n = 3–6).

B. RT-PCR using primers located in exons 4 and 9 of Mybpc3 or in Gapdh. Total Mybpc3 mRNA level was normalized to Gapdh and related to WT. Determination of the mRNA levels was performed by densitometry (Gene Tool Software; Syngene, Cambridge) and Var-4 Mybpc3 mRNA level was expressed as a percentage of total Mybpc3 mRNA. Data are expressed as mean ± SEM. ***p < 0.001 versus PBS-treated WT, and ###p < 0.001 versus PBS-treated KI, one-way ANOVA and Bonferroni post hoc test. Number of animals is indicated in the bars.

C. Western blot stained with antibodies directed against the MyBP-C motif of cMyBP-C or α-actinin. BW, body weight; FAS, fractional area shortening; LVM, left ventricular mass; M, 100-bp molecular weight marker.
increased Var-4 mRNA level as early as 7 days after injection (Fig 8). Var-4 expression was persistent for 22–25 days (Fig 5) and 55 days (Fig 9) post-injection in KI mice. These data provide evidence that AONs directed against ESEs of exon 5 and/or exon 6 are sufficient to produce a “therapeutic mRNA”. This is accompanied by markedly lower Mut-1 or Mut-1 plus Mut-3 mRNAs. It is well perceivable that efficient exon skipping replaced non-functional Mut-1/Mut-3 by Var-4 cMyBP-C protein. We also provide evidence that U7snRNA-mediated exon skipping rescued the cardiomyopathic phenotype in newborn KI mice 7 days after injection (Fig 7). The finding that AAV9-Var-4 systemic delivery similarly restored cardiac function (Fig S7) supports the view that Var-4 is functional and non toxic for the heart, and at least in part, the reason for the functional rescue in KI mice. The rescue of the phenotype was much less obvious 55 days after administration of AAV9-U7-AON5 + 6 in newborn KI mice (Fig 9). On the one hand, AAV-dosing may play a role. This is supported by the observation of a 4-fold lower number of virus particles in the heart 55 days than 7 days after AAV9 injection (Fig S6). The drop of virus particles in the first month after injection is in agreement with recent data (Hu & Lipshutz, 2012). This indicates the need to inject even a higher dose from the beginning or to inject later another dose of AAV. On the other hand, the different state of the diseased heart and developmental changes could also be relevant. It is apparent, for instance, that FAS went down over time in WT mice (from 53 to 43%), whereas it formally increased in KI mice between days 12–14 and 55–57 (Fig 9). The reasons are unknown at present, but it is apparent that LVM and FAS evolved similarly over time in AON-5+6-treated KI mice and WT (at a lower level) and clearly differed from PBS-treated KI. This in fact suggests a persistent therapeutic effect, which would be in line with the persistent molecular rescue. In addition, whereas 4-week-old KI mice showed marked hypertrophy (thickening of walls) in addition to LV dilation, newborn KI mice only exhibited LV dysfunction and dilation, and marked activation of Nppa and Nppb genes (Fig 6A,B). Development of LVH started at postnatal days 3-4 and was associated with the activation of Acta1 expression, but not of Myh7, which actually remains high in KI compared to WT (Fig 6C). These findings are interesting as they indicate that dysfunction (systolic and likely also diastolic) precedes LVH in (this model of) HCM. Similar observations were made in 2 other mouse models of HCM (homozygous Myh6- and Mybpc3-targeted KI mice; Fatkin et al, 1999; McConnell et al, 1999), and in a human patient carrying a homozygous MYBPC3 mutation, who died at the age of 9 month from a marked LV dilation and dysfunction (Richard et al, 2003).

In summary, this proof-of-principle study showed for the first time that AONs targeting ESEs in mouse Mybpc3 induces the skipping of the corresponding exons and results in a modified in-frame mRNA and protein in vitro and in vivo. Consequently, systemic administration of AAV9 encoding U7-AON5+6 was sufficient to abolish cardiac dysfunction and to prevent development of LVH in newborn KI mice. Although further optimization is needed to maintain the therapeutic cardiomyopathic rescue over an extended period, our findings paves the way for a causal therapy of HCM.

MATERIALS AND METHODS

Mybpc3-targeted knock-in mice
The investigation conforms to the NIH guidelines for the care and use of laboratory animals published by the NIH (Publication No. 85–23, revised 1985). The experimental procedures were in accordance with the German Law for the Protection of Animals and accepted by the Ministry of Science and Public Health of the City State of Hamburg, Germany (Nr: 69/10). Development and initial characterization of the Mybpc3-targeted KI mouse model was previously reported (Vignier et al, 2009). In brief, KI mice carry a C>A transition on the last nucleotide of exon 6, which was introduced by gene targeting using the Cre/lox system (Vignier et al, 2009). WT and KI mice were maintained on the Black Swiss genetic background.

Culture and transfection of HEK293 cells
HEK293 cells were maintained at 37°C and 7% CO2 in Dulbecco’s modified Eagle’s medium supplemented with 10% heat inactivated foetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). For transient transfections cells were plated at a density of 2.5 × 10⁵ cells in 12-well dishes and incubated until a confluency of 50–70% Transfection with 2 µg plasmid DNA was conducted using TurboFect® (ThermoScientific) following the instructions of the manual. After 48 h cells were cultured for 15 min in the presence or absence of forskolin (10 µM) and 3-isobutyl-1-methylxanthine (IBMX, 250 µM), which increases cAMP level and therefore activate PKA. DMSO was used as a negative control. Cells were then harvested for RNA and protein analysis.

AON design
AON-5 (5’-CCA GCC ACU CGG GCU GAG AAG ACA A-3’) and AON-6 (5’- AAG UGC UCU GAC CAU CUG UGA UGU G-3’) target ESE motifs in exon 5 or exon 6 of Mybpc3, respectively. The motifs were identified using the ESE prediction program “ESE finder 3.0” (Cartegni et al, 2003; Smith et al, 2006). To test the specificity of the AONs (25-mers) 2′-O-methyl RNA with a full-length phosphoroethioate 2OMePS backbone was synthesized (Eurogentec).

Culture and transfection of neonatal mouse cardiac myocytes
NMCMs were isolated from up to 25 hearts of 0-4 day-old pups and cultured as described previously (Vignier et al, 2009). After 3 to 4 days of plating cytosine arabinoside (Ara-C) was added (25 µM) to prevent division of remaining fibroblast cells. WT or KI NMCMs were transfected with 5 µg modified AON-5 or AON-5+6. The most effective AON quantity was determined in a pre-test. TurboFect™ was used for all transfections according to the manufacturer instructions. Non-transfected, but transfection reagent-containing (mock) NMCMs served as controls.

Production and transduction of adeno-associated viral vectors in vitro and in vivo
For AAV production the self-complementary vector pAAVscU7 and the HEK293-AAV cell line (Cell Biolabs) were used. Cells were maintained at
37°C and 5% CO₂ in Dulbecco’s modified Eagle's medium supplemented with 10% heat inactivated foetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). Both AON-5 and AON-6 were inserted in separate cassettes each containing the modified U7snRNA gene along with its natural promoter and 3’ elements. Both cassettes were then cloned in tandem into the XbaI and AarI sites in pAAVsCU7. For production of AAV6 pseudotyped vectors a double transfection of pAAVsCU7-AON-5+6 snRNA and pDD6 (kindly provided by J. Kleinschmidt, Heidelberg) encoding adenosivirus helper functions, AAV2 rep and AAV6 cap genes in HEK293T cells were conducted (Grimm et al., 2003; Muller et al., 2006). AAV9 pseudotyped vectors were prepared by triple-transfection of pAAVsCU7-AON-5+6 snRNA, pXX6 encoding adenosivirus helper functions and pAAV2-9 (RepCap) in HEK293 cells. Vector particles were purified 72 h after transfection on iodixanol gradients from cell lysates. Gradient fractions were concentrated using centrifugal cartridges (Amicon ultra-4 50K or ultra-15 100K), whereby iodixanol was exchanged against PBS or PBS-MK (Gibco; supplemented with 1 mM MgCl₂ and 2.5 mM KCl). AAV6 titeres were determined in triplicates by qPCR using the Maxima™ SYBR Green/Rox qPCR Master Mix (ThermoScientific) and primers complementary to vector sequences (forward: 5′-ACT CGG CCT GCT GAG AAG ACA AAA TTT TTG GAG CA-3′; reverse: 5′-GAG TGG CCA GGC GAG GAC-3′). AAV9 titeres were determined in triplicates by qPCR using a Taqman-specific probe (forward: 5′-CTC CAT CAC TAG GGG TTC CTT CT-3′; reverse: 5′-GTA CAT AAG TAG CTT GGC-3′; probe: 3′-TAG TTA ATG ATT AAC CC-5′). Prior to titration viral DNA was extracted by diluting the virus 1:10 in TE buffer (20 mM Tris, 2 mM NaCl, 0.5 mM EDTA) for 10 min at room temperature. The supernatants were collected and protein concentration determined using the Bradford protein assay (BioRad). Some samples were extracted with Trizol® (Invitrogen) followed by pellet solubilization in 8 M Urea in 50 mM Tris, pH 7.4. Proteins (HEK293 cells: 0.5–30 μg; murine tissues: 7.5–15 μg) were either loaded on 6–10% acrylamide/bisacrylamide (29:1) gels or 4–12% NuPAGE® Bis-Tris gels (Life Technologies) and electrotransferred on nitrocellulose membranes (Whatman®). Membranes were incubated overnight with primary antibodies (polyclonal antibodies directed against Ser-282-cMyBP-C (1:1000; custom made, Ei-Armouche et al., 2007), the MyBP-C motif of cMyBP-C (1:1000; gift from C. Witt, Mannheim, Germany), 2-14aa- (1:15,000), Ser273- (1:2000) and Ser302-cMyBP-C (1:10,000; gifts from S. Sadayappan, Chicago, IL); Var-4-cMyBP-C (1:1000; custom made), or total ERK (p44/42 MAPK; 1:1000; Cell Signaling); monoclonal antibodies directed against α-actinin (1:1000, Sigma) or the FLAG epitope (1:5000, Sigma). The membranes were washed and incubated with peroxidase-labeled secondary antibodies (anti-rabbit IgG 1:5000, Dianova or anti-rabbit IgG 1:6000, Sigma). Protein bands were visualized using the SuperSignal® West Dura (Pierce) according to manufacturer’s instructions. Signals were detected with the Chemie Genius™ Bio Imaging System and quantified with the Gene Tool Software (Syngene, Cambridge). Obtained values were plotted using the software GraphPad Prism 5 (GraphPad Software, Inc.).
PROBLEM:
Exon skipping mediated by AONs is a promising therapeutic approach for selected genetic disorders, but has not yet been evaluated for cardiac genetic diseases. Hypertrophic cardiomyopathy (HCM) is often caused by mutations in MYBPC3 encoding cardiac myosin-binding protein C. The study investigated the feasibility and efficacy of viral-mediated AON transfer in a Mybpc3-targeted knock-in (KI) mouse model of HCM.

RESULTS:
KI mice carry a homozygous G>A transition in exon 6, which results in 3 different aberrant mRNAs and/or proteins. In addition, we identified an alternative variant (Var-4) deleted of exons 5–6 in wild-type and KI mice. To enhance its expression and suppress aberrant mRNAs we designed AON-5 and AON-6 that mask ESE motifs in exons 5 and 6. AONs were inserted into modified U7 small nuclear RNA and packaged in adeno-associated viral vectors (AAV-U7-AON-5 + 6). Treatment with AAV-U7-AON-5 + 6 markedly increased Var-4 mRNA and protein levels and reduced aberrant mRNAs. Systemic administration in newborn KI mice restored cardiac function and prevented left ventricular hypertrophy.

IMPACT:
The present study provides the first proof-of-principle evidence that AAV-U7-AONs remove a mutation in neonatal mouse cardiac myocytes and in vivo in the heart of a HCM mouse model. AON-mediated exon-skipping increased the amount of the internally deleted but functional cMyBP-C variant. Cardiomyopathy was rescued through U7snRNA-mediated exon skipping in neonatal mice. Therefore, our findings laid the first stone towards a causal therapy of HCM.

For more information
DZHK (Deutsches Zentrum für Herz-Kreislauf-Forschung e.V., DZHK): German Centre for Cardiovascular Research
http://dzhk.de/
BIG-Heart (Health-F2-2009-241577): European network on hypertrophic cardiomyopathy
http://www.big-heart.eu/
Genethon: Biotherapies Institute
http://www.genethon.fr/en/
Institut de Myologie
http://www.institut-myologie.org/

Echocardiography
Transthoracic echocardiography was performed using the Vevo 2100 System (VisualSonics, Toronto, Canada). Animals were anaesthetized with isoflurane (1–2%) and assured to a warming platform in a supine position. B-mode images were obtained using a MS400 transducer for adult mice and a MS550 transducer for neonatal mice. For 4-week-old mice echocardiography was performed prior tail-vein injection and then weekly, and for newborn mice 7 days after injection. Images were obtained in a parasternal short and long axis view. The dimensions of the left ventricle (thickness of the septum and posterior wall, as well as left ventricular diameter) were measured in a short axis view in diastole and systole.

Statistical analysis
Data were expressed as mean ± SEM. Statistical analyses were performed using the unpaired Student’s t-test or the one-way or two-way ANOVA followed by Dunnett or Bonferroni post-hoc test using the commercial software GraphPad Prism5 (Software Inc.). A value of p < 0.05 was considered statistically significant.
contributed to the design of modified AONs; LG advised experiments with AONs; LC designed and supervised the entire study, obtained funding and finalized the manuscript. All authors critically reviewed and approved the manuscript before submission.

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Supporting Information is available at EMBO Molecular Medicine Online.

The authors declare that they have no conflict of interest.

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