Original Research

Allele-specific silencing by RNAi of R92Q and R173W mutations in cardiac troponin T

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Impact Statement

Allele-specific silencing represents a promising therapeutic strategy for human cardiomyopathies. A major critical step is siRNA design and validation to obtain highest silencing effect with best allele discrimination. We report here on the identification and validation of siRNA sequences to knockdown mutant alleles coding for the R92Q and the R173W missense mutations in human cardiac troponin T (*TNNT2*) gene. siRNAs were validated by a two-step protocol consisting in the use of a luciferase reporter assay followed by transfection in HEK293T cells and the western blot analysis. The results obtained showed that insertion of a single-base mismatch downstream the targeted mutations conferred the highest allele-specific discrimination, with minor effects on expression of the wild-type allele, suggesting that this approach can potentially be considered for further validation in cardiac muscle cells.

Abstract

Autosomal dominant mutations in sarcomere proteins such as the cardiac troponin T (*TNNT2*) are the main genetic causes of human hypertrophic cardiomyopathy and dilated cardiomyopathy. Allele-specific silencing by RNA interference (ASP-RNAi) holds promise as a therapeutic strategy for downregulating a single mutant allele with minimal suppression of the corresponding wild-type allele. Here, we propose ASP-RNAi as a possible strategy to specifically knockdown mutant alleles coding for R92Q and R173W mutant TNNT2 proteins, identified in hypertrophic and dilated cardiomyopathy, respectively. Different siRNAs were designed and validated by luciferase reporter assay and following analysis in HEK293T cells expressing either the wild-type or mutant *TNNT2* alleles. This study is the first exploration of ASP-RNAi on *TNNT2*-R173W and *TNNT2*-R92Q mutations *in vitro* and gives a base for further application of allele silencing as a therapeutic treatment for *TNNT2*-mutation-associated cardiomyopathies.

Keywords: RNA interference, cardiomyopathy, luciferase assay, gene therapy

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Introduction

Cardiomyopathies (CMPs) are myocardial disorders characterized by structural and functional abnormalities in the cardiac muscle.[1] Many familial forms of hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM) are linked to autosomal dominant mutations in genes that encode thick and thin contractile myofilament proteins of the cardiac sarcomere, and both represent a significant risk of heart failure and sudden death for the affected individuals.[2,3] HCM affects 1:500 in the population and is characterized by a left ventricular hypertrophy with diastolic dysfunction, whereas DCM, with the prevalence of 1:2500, is characterized by a dilated left ventricular or biventricular cavity and manifests as systolic dysfunction.[4,5] HCM is associated with sudden cardiac death only in a small percentage of subjects, especially in young men, while most of the patients have mild symptoms. However, recent studies show that heart failure, left ventricle fibrosis, and atrial dilatation in HCM patients can be an important cause of morbidity and mortality in the long term. On the contrary, DCM represents one of the main causes of heart transplantation and is associated with an increased risk of heart failure, with significant morbidity and mortality as well as arrhythmia-related death.[6]

The human *TNNT2* gene codes for TNNT2, the largest troponin subunit involved in the regulation of cardiac contraction in response to calcium concentration.[7,8] Pathogenic mutations in *TNNT2* account for approximately 15% of HCM[9] and 2.9% of DCM cases.[10,11] Of note, mutations in genes coding for proteins of the troponin complex appear to be particularly severe and penetrant in both types of CMPs.[6] Among them, the R92Q missense mutation (c.275

G > A; p.R92Q) has been identified as causative for HCM and it is associated with a particularly poor clinical prognosis,[12,13] whereas the R173W mutation (c.517, C > T; p.R173W) is linked to DCM.[14] Both are involved in alteration of the Ca^{2+} sensitivity of the troponin complex and of the binding affinity of *TNNT2* for tropomyosin.[15,16] Current cardiomyopathy treatments focus on symptoms relief by pharmacological intervention and prevention of sudden cardiac death. Several therapeutic approaches, including the allele-specific silencing (ASP-RNAi), have been proposed to prevent CMP development, correcting the mutation before the beginning of symptoms.[17] Recently, ASP-RNAi of the mutant allele with specific siRNAs has been reported in some autosomal dominant diseases.[18–21] The potential of ASP-RNAi is largely dependent on its highly sequence-specific knockdown ability; to this purpose, it is necessary to design siRNAs that confer a strong allele discrimination. An important issue in designing efficient siRNAs is the position of the mutant nucleotide in the siRNA sequence; indeed, different studies demonstrated that siRNAs that carry the mutant nucleotide in a central position show an enhanced allele discrimination and display a better performance in cleavage of the target transcript.[22–25] In addition, introduction of additional modification can further improve allele-specific silencing.[25] Among these, the most effective modification appears to be the introduction of nucleotide mismatches into siRNA duplexes,[26,27] although the nucleotide position and the choice of the base are critical aspects to be evaluated. Accordingly, mismatches can be introduced in the 3′-end of sense siRNA sequence, containing the seed region, resulting in an increase in allele specificity thanks to a significant decrease in base-pairing efficiency between siRNA and the wild-type allele.[28]

In this study, we set up the basis for development of an ASP-RNAi approach to specifically knockdown mutant alleles coding for the R92Q and the R173W missense mutations in *TNNT2* gene. Since siRNA design is one of the most critical steps in ASP-RNAi, we designed and tested sets of siRNAs fully complementary to the target sequence of the mutant alleles or containing single-base mismatches downstream the targeted mutations. Competent siRNAs conferring allele-specific silencing against the R92Q and R173W mutant alleles were selected and validated using a luciferase assay. The efficiency of allele-specific knockdown was confirmed in cells expressing full-length wild-type or mutant *TNNT2*.

Materials and methods

Design of siRNAs and construction of reporter and expression plasmids

Lyophilized silencing RNA oligonucleotides (siRNAs) were purchased from Merck (Kenilworth, NJ, USA) and resuspended in the provided buffer at a final concentration of 20 µM. In each experiment, they were used at a final concentration of 200 nM. Non-silencing siRNA duplex (Ambion, Austin, TX, USA) was used as a negative control. Sequences of the siRNAs used are indicated in Figures 1(a) and 2(a).

To construct the luciferase reporter plasmids, the phRL-TK (Promega, Madison, WI, USA) and pGL3-TK (kindly provided by Professor H. Hohjoh, Tokyo, Japan[29]) plasmids containing the coding sequences of *Renilla reniformis* and *Photinus pyralis* luciferases, respectively, both under the control of the herpes simplex virus thymidine kinase (TK) promoter, were used. Oligonucleotide sequences corresponding to selected regions of human wild-type and mutant *TNNT2* (NM_001001430.3) were designed and chemically synthesized (Merck); the SphI restriction site for selection of positive clones and the XbaI and NotI restriction sites for cloning steps were included. Oligonucleotides were annealed in annealing buffer composed by 100 mM Tris-HCl (pH 8), 10 mM EDTA, 1 M NaCl to the final concentration of 100 µM, and cloned in the 3′-untranslated region (UTRs) of the luciferase genes. In detail, mutant *TNNT2* sequences were inserted in the pRL-TK plasmid and WT *TNNT2* sequences were cloned in the pGL3-TK.

Cell line

293T human embryonic kidney cells (HEK293T) were grown in Dulbecco's modified Eagle's medium (DMEM) (Merck) supplemented with 10% heat inactivated fetal bovine serum (Merck), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM l-glutamine, and 1 mM sodium pyruvate at 37°C in a 5% CO_2 humidified chamber.

Transfection and luminescent assays

The day before the transfection HEK293T cells were detached by trypsin-EDTA (0.05% trypsin, 0.53 mmol/L EDTA·4Na) at 70–90% confluency, resuspended in fresh medium and seeded into 24-well culture plates at a density of 0.5×10^5 cells/well to perform luciferase reporter assay or into 60 mm plates at the density of 1×10^6 cells/well to perform the western blot analysis. Transfections with synthetic siRNA duplexes and reporter plasmids were carried out using Lipofectamine Transfection Reagent (Thermo Fisher, Waltham, MA, USA) and Oligofectamine Reagent (Thermo Fisher) according to the manufacturer's instructions. Before co-transfection, the culture medium was replaced by antibiotics and serum free DMEM. Cells were transfected with 0.2 µg of luciferase reported plasmid, 0.1 µg of pSV-β-galactosidase control vector (Promega) and 200 nM of siRNA duplexes. Cells were incubated for 3 h at 37°C and then fresh culture medium supplemented with 20% of fetal bovine serum was added. Cells were incubated at 37°C for 24 h. Cells were lysed using passive lysis buffer (Promega) and the expression levels of luciferase and β-galactosidase were analyzed by the Dual-Luciferase Reporter Assay System (Promega) and Beta-Glo Assay System (Promega). 20 µL of cell lysate was transferred into a luminometer tube containing the Luciferase Assay Reagent II to generate a stabilized luminescent signal form *Photinus pyralis* luciferase. The *Renilla reniformis* luciferase reaction is simultaneously initiated by adding Stop & Glo Reagent to the same tube. 20 µL of cell lysate was used to measure the β-galactosidase activity by the addition of the Beta-Glo Reagent. The luminescence intensity of both luciferases and β-galactosidase was determined by a luminometer (TD20/20, Promega), and the levels of activity of either mutant or wild-type luciferases were normalized to the levels of β-galactosidase activity. The ratio of mutant and wild-type luciferase activities in the presence of siRNAs targeting the *TNNT2* mutant alleles was normalized to the

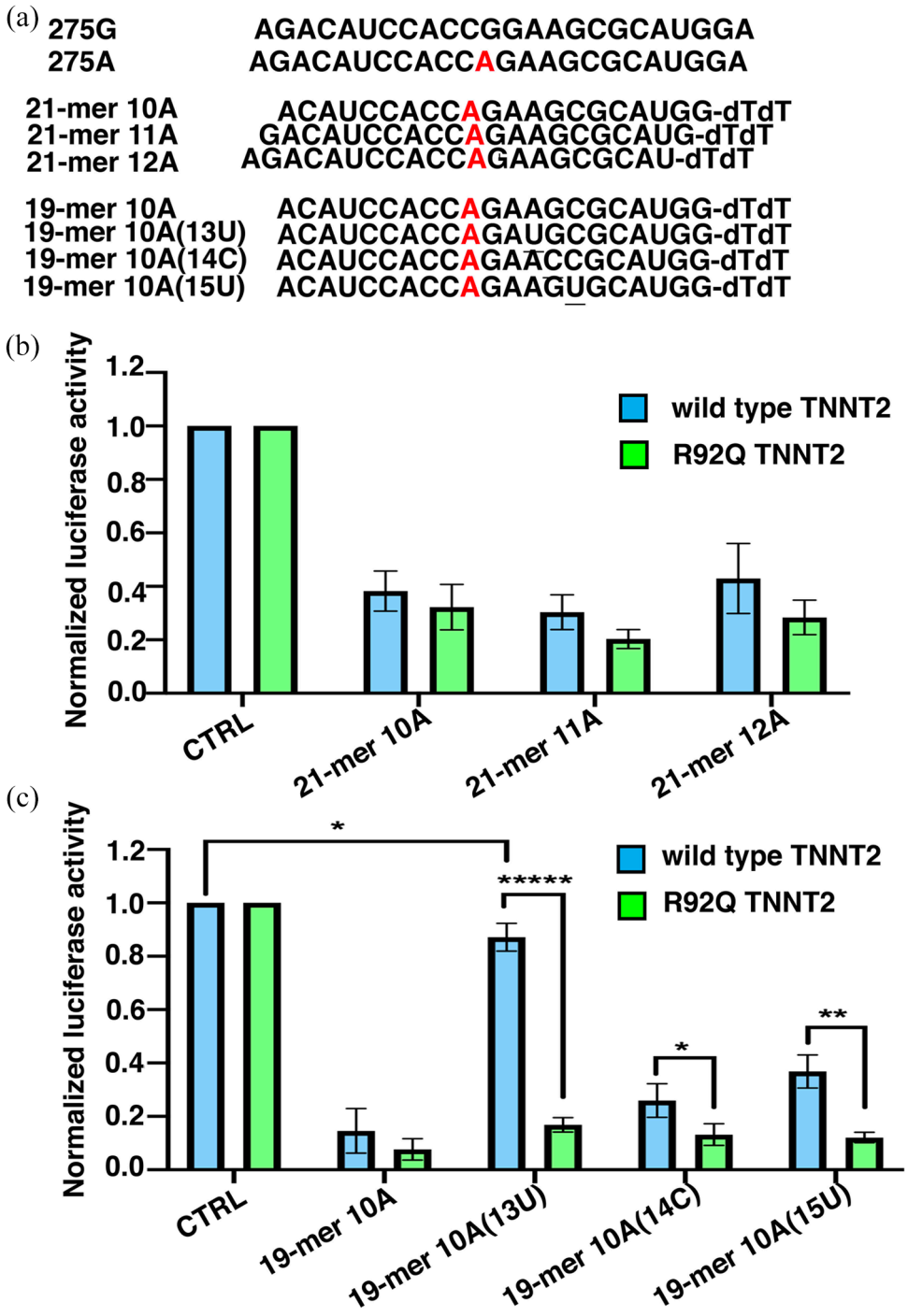

Figure 1. Assessment of ASP-RNAi on luciferase reporter plasmids. (a) siRNA duplexes targeting the R92Q mutation: 21-mer sense-strand siRNAs (10A–12A), 19-mer sense-strand 10A siRNA, and 19-mer sense-strand 10A siRNAs carrying a mismatch in the seed region. The A nucleotide substitution resulting in the R92Q amino acid change is shown in red, whereas the additional mismatches are underlined. (b and c) Levels of luciferase activity obtained in HEK293T cells co-transfected with the reporter plasmids for the R92Q or the wild-type *TNNT2* alleles were normalized to the levels of β-galactosidase activity. β-galactosidase-normalized luciferase activity in the presence of siRNA duplexes was normalized to the activity measured in the presence of control siRNA. Data are presented as mean value ± SD of four independent experiments (*$P < 0.05$; **$P < 0.005$; *****$P < 0.000005$). (A color version of this figure is available in the online journal.)

ratio obtained in the presence of control siRNA. Statistical analysis was performed by Student's *t*-test analysis.

Construction of wild-type and mutant *TNNT2* expression plasmids

To construct full-length *TNNT2* expression plasmids, the TNNT2 cDNA (NM_001001430.3) was retrotranscribed and amplified by polymerase chain reaction (PCR) (forward primer, 5′-CCCGCTGAGACTGAGCAGA-3′ and reverse primer, 5′-AGGCCAGCTCCCCATTTCC-3′) from total human heart RNA and inserted in pGEM-T Easy vector (Promega). The pGEM-T Easy-TNNT2 vector obtained was digested with EcoRI (Promega) restriction enzyme and inserted into pcDNA3 vector (Invitrogen, Waltham, MA, USA). The

resultant plasmid was designated "pcDNA3-WT-TNNT2." Starting from this plasmid, mutant plasmids carrying the TNNT2 R92Q and R173W substitutions were constructed by site directed mutagenesis and designated "pcDNA3-R92Q-TNNT2" and "pcDNA3-R173W-TNNT2."

Western blot analysis

HEK293T cells were collected 24 h after transfection, washed twice with ice-cold PBS (Merck), harvested by centrifugation at 16,000*g* for 15 min at 4°C and lysed in lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, and 0.25% sodium deoxycholate) supplemented with protease inhibitors cocktail and 0.1 mM PMSF (phenylmethylsulphonyl fluoride). Lysed cells were collected and incubated for 1 h at 4°C and subsequently sonicated (30 pulses, 30% power: Bandelin Sonopuls). Protein concentration was measured using Protein Assay Dye Reagent Concentrate kit (Bio-Rad, Hercules, CA, USA) at Ultraspec 2100 pro UV–Visible Spectrophotometer (Amersham Bioscience, UK). Equal amounts of protein (100 µg) were mixed with 4× sample buffer (250 mM Tris-HCl pH 6.8, 8% SDS, 40% glycerol, 20% beta-mercaptoethanol, and 0.016% bromophenol blue), boiled for 5 min at 95°C and then separated by SDS-PAGE on 10% polyacrylamide gel using TGS running buffer (25 mM Tris, 190 mM glycine, and 1% SDS) at 25 mA for 90 min at room temperature. After electrophoresis, proteins were blotted onto nitrocellulose blotting membrane (GE Healthcare, Life science, Chicago, IL, USA) using a transfer buffer (25 mM Tris, 190 mM glycine, 15% methanol, and 0.1% SDS) at 400 mA for 2 h in a cold room. The membrane was incubated for 1 h in blocking solution with 5% Bovine Serum Albumin (BSA) (Merck) in TBS-T (150 mM NaCl, 10 mM Tris, pH 7.4, 0.2% Tween-20) and then diluted with primary antibodies (described below) at 4°C overnight; membranes were washes in TBS-T buffer, and further incubated with 1:3000 diluted horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Amersham) for 1 h at room temperature. Antigen-antibody complexes were visualized using Immobilon Western Chemiluminescent HRP Substrate (Bio-Rad) and the image was acquired with Chemidoc MP (Bio-Rad). The intensity of the bands was quantified using Image Lab™ Software (Bio-Rad). The primary antibodies used in western blotting and their dilution ratios were as follows: monoclonal anti-TNNT2 antibody (1:200) (Invitrogen) and monoclonal anti-GFP (1:1000) (Merck) antibody.

Results

ASP-RNAi of the c.275 G > A variant coding for the R92Q mutant TNNT2 protein

To identify competent allele-specific siRNAs, the dual luciferase reporter assay was used. To this aim, a 37-nucleotide fragment from either human wild-type or R92Q mutant *TNNT2* sequence were cloned into the 3′-UTR of the genes coding for *Photinus pyralis* and *Renilla reniformis* luciferases, respectively (Supplemental Figure S1A). To test the ability of different siRNA to specifically silence the wild-type or the R92Q mutant alleles, HEK293T cells were co-transfected with the two reporter plasmids and with each siRNA duplex to be tested. An unrelated siRNA duplex was also used as control.

A first screening was performed to analyze three 21-mer siRNAs fully matching the sequence of the mutant *TNNT2* and carrying the c.275 G > A substitution at positions +10, +11, or +12 of their sequence (Figure 1(a)). All siRNA duplexes analyzed inhibited the expression of both luciferases indicating that they were not able to exert a statistically significant allele-specific silencing. In detail, the residual expression of mutant *TNNT2* allele ranged from 32 ± 8.5% to 20 ± 3.6% and that of the wild-type *TNNT2* allele from 43 ± 13% to 30 ± 6.5% as compared to control siRNA (Figure 1(b)). Since the tested siRNAs did not induce any ASP-RNAi, we thought to improve the allele specificity by introducing of a single-base mismatch.[27] Among the three siRNAs analyzed, the 21-mer 12A siRNA was shown to exert the best allele-specific effect.

We thus designed three novel 19-mer siRNAs containing the G > A substitution at position 10 and a single-base mismatch in the seed region (A > U, G > C, and C > U at positions 13, 14, and 15, respectively) (Figure 1(a)). As control, a 19-mer siRNA containing only the G > A substitution was also tested (19-mer 10A). siRNAs were tested by luciferase assay and the effects on luciferase activity of the 19-mer siRNAs are shown in Figure 1(c).

The results obtained showed that the 19-mer 10A siRNA induces a more significant silencing effect of the mutant allele (92.4 ± 4%), compared to the 21-mer 12A siRNA (79.7 ± 3.6%), although it still shows only a partial allele discrimination. Analysis of the 19-mer 10A siRNAs containing single-base mismatches showed that the 19-mer 10A (13U) siRNA induced the more efficient allele discrimination, with a silencing of the mutant allele of 83.1 ± 2.7% and of 12.8 ± 5.2% of the wild-type allele. In contrast, 19-mer 10A (14C) and 10A (15U) siRNAs although showing a significant allele specificity, they also resulted in a strong reduction in the expression of the wild-type allele.

ASP-RNAi of the c.517 C > T variant coding for the R173W mutant TNNT2 protein

To induce ASP-RNAi of mRNA containing the *TNNT2* R173W mutation, four 19-mer siRNA duplexes complementary to the human *TNNT2* sequence carrying the c.517 C > T mutation at position +9, +10, +11, and +12 were designed (Figure 2(a)). Silencing efficiency and allele discrimination were evaluated by the dual luciferase reporter assay. As for the R92Q mutation, a 37 nucleotides fragment from either human wild-type or R173W mutant *TNNT2* sequences were cloned in the 3′-UTR of the genes coding for *Photinus pyralis* and *Renilla reniformis* luciferases (Supplemental Figure S1B).

The luciferase assay showed that siRNA 9U, 11U, and 12U achieved a significant ASP-RNAi; in detail, siRNA 9U and 12U induced the strongest silencing of the mutant allele (Figure 2(b)), with an inhibition of 95.6 ± 1.8% for the siRNA 9U and 93.2 ± 2.3% for the siRNA 12U. To improve siRNA specificity for the mutant allele, we designed additional siRNA including a mismatch in the seed region of siRNA 9U and siRNA 12U. We thus designed siRNA 9U (13U), 9U (14C), and 9U (15U) carrying a base mismatch in the seed

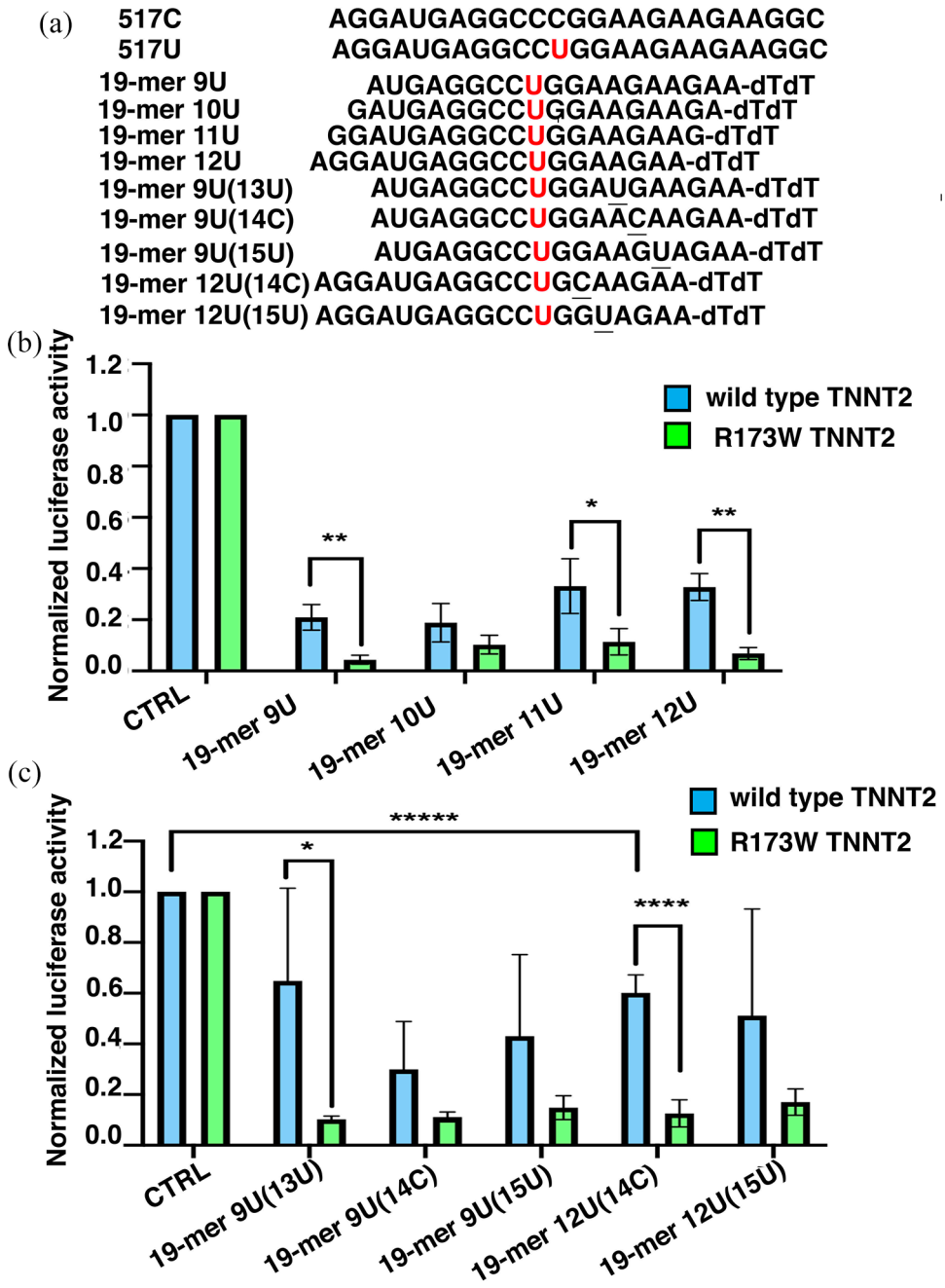

Figure 2. Assessment of ASP-RNAi on reporter alleles. (a) siRNA duplexes targeting the R173WQ mutation: 19-mer sense-strand siRNA and 19-mer sense-strand siRNAs carrying a mismatch in the seed region. The C nucleotide substitution resulting in the R173W amino acid change is shown in red, whereas the additional mismatches are underlined. (b and c) Levels of luciferase activity obtained in HEK293T cells co-transfected with the reporter plasmids for the R173W or the wild-type *TNNT2* alleles were normalized to the levels of β-galactosidase activity. β-galactosidase-normalized luciferase activity in the presence of siRNA duplexes was normalized to the activity measured in the presence of control siRNA. Data are presented as mean value ± SD of four independent experiments (*$P < 0.05$; **$P < 0.005$; *****$P < 0.000005$). (A color version of this figure is available in the online journal.)

region (A > U, G > C, and A > U in positions 13, 14 and 15, respectively). Similarly, siRNAs 12U (14C) and 12U (15U) were designed based on siRNA 12U sequence and including a single-base mismatch in the seed region (G > C and A > U, respectively) (Figure 2(a)). Results from luciferase assay showed that the introduction of a single-base mismatch resulted in a significant improvement of mutant allele silencing in comparison to the wild-type allele. In detail, among the five siRNAs tested, the 12U (14C) induced a strong ASP-RNAi, with 87.4 ± 5.3% knockdown of the R173W allele against a 39.9 ± 7.1% decrease in expression of the wild-type allele (Figure 2(c)).

Analysis of ASP-RNAi on full-length wild-type and mutant TNNT2 protein expression

To analyze the silencing effect of selected siRNA duplexes on expression of wild-type and mutant TNNT2 proteins, we co-transfected HEK293T cells with siRNAs 19-mer 10A (13U) and plasmids coding for either full-length wild-type or R92Q

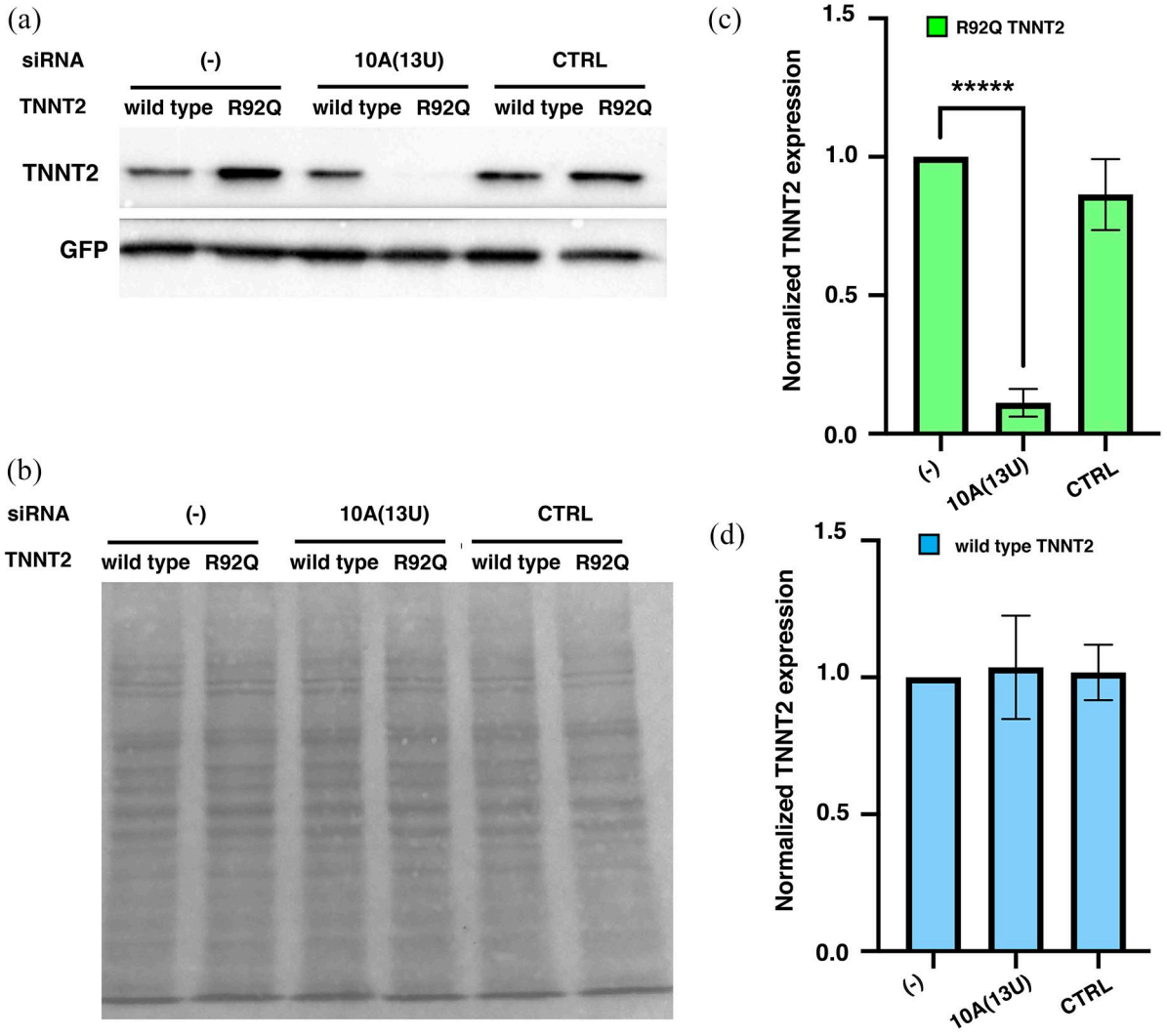

Figure 3. siRNA 10A (13U) silences the expression of R92Q TNNT2 protein. (a) Representative western blot analysis of whole lysates of HEK293T cells co-transfected with expression plasmids coding for full-length wild-type or R92Q TNNT2 and control (CTRL) or 10A (13U) siRNAs. Protein extracts of cells transfected only with TNNT2 expression plasmids were used as control (–). Transfection efficiency was evaluated by co-transfection with a GFP expression plasmid. (b) Ponceau-red staining of nitrocellulose membrane following protein transfer, related to western blot in (a). (c) R92Q TNNT2 relative expression in control cells (–) and in cells transfected with either siRNA 10A (13U) or control siRNA (CTRL). (d) Wild-type TNNT2 relative expression in control cells (–) and in cells transfected with either siRNA 10A (13U) or control siRNA (CTRL). Data are presented as mean value ± SD of three independent experiments (*****$P < 0.0000005$). (A color version of this figure is available in the online journal.)

mutant TNNT2 proteins. Alternatively, HEK293T cells were co-transfected with siRNAs 19-mer 12U (14C) and plasmids coding for either full-length wild-type or R173W mutant TNNT2 proteins. The silencing effect of mutation-specific siRNA was thus evaluated on total protein lysates expressing either wild-type or mutant proteins.

Expression of siRNA 19-mer 10A (13U) resulted in a decrease of 88.8 ± 4.9% of R92Q TNNT2 protein expression compared to cells co-transfected with control siRNA or not transfected with any siRNA (Figure 3(a) and (c)). siRNA 19-mer 10A (13U) also showed a significant allele-specific silencing effect since no significant decrease in the expression of the wild-type TNNT2 protein was observed (Figure 3(d)). Similarly, the western blot analysis of whole cell lysates from cells transfected with siRNA 12U (14C) showed a strong (92.7 ± 4.2%) silencing of R173W TNNT2 protein expression (Figure 4(a) and (c)). However, in contrast to 19-mer 12A (13U), the 12U (14C) siRNA also induced a significant decrease of the wild-type TNNT2 protein expression (Figure 4(d)).

Discussion

We report here on the identification and validation of siRNA sequences to knockdown mutant alleles coding for the R92Q and the R173W missense mutations in human *TNNT2* gene. Heterozygous dominant mutations in sarcomere proteins of the thick and thin filaments are the main genetic cause of HCM and DCM. The troponin T sarcomere protein is the largest component of the troponin complex. It is involved in the regulation of cardiac muscle contraction and is responsible for the association of the troponin complex with the thin filament and for the positioning of the other troponin subunits in the complex.[7] Most of DCM and HCM-causing mutations in *TNNT2* gene lead to the expression of mutant proteins that are incorporated in the sarcomere and affect

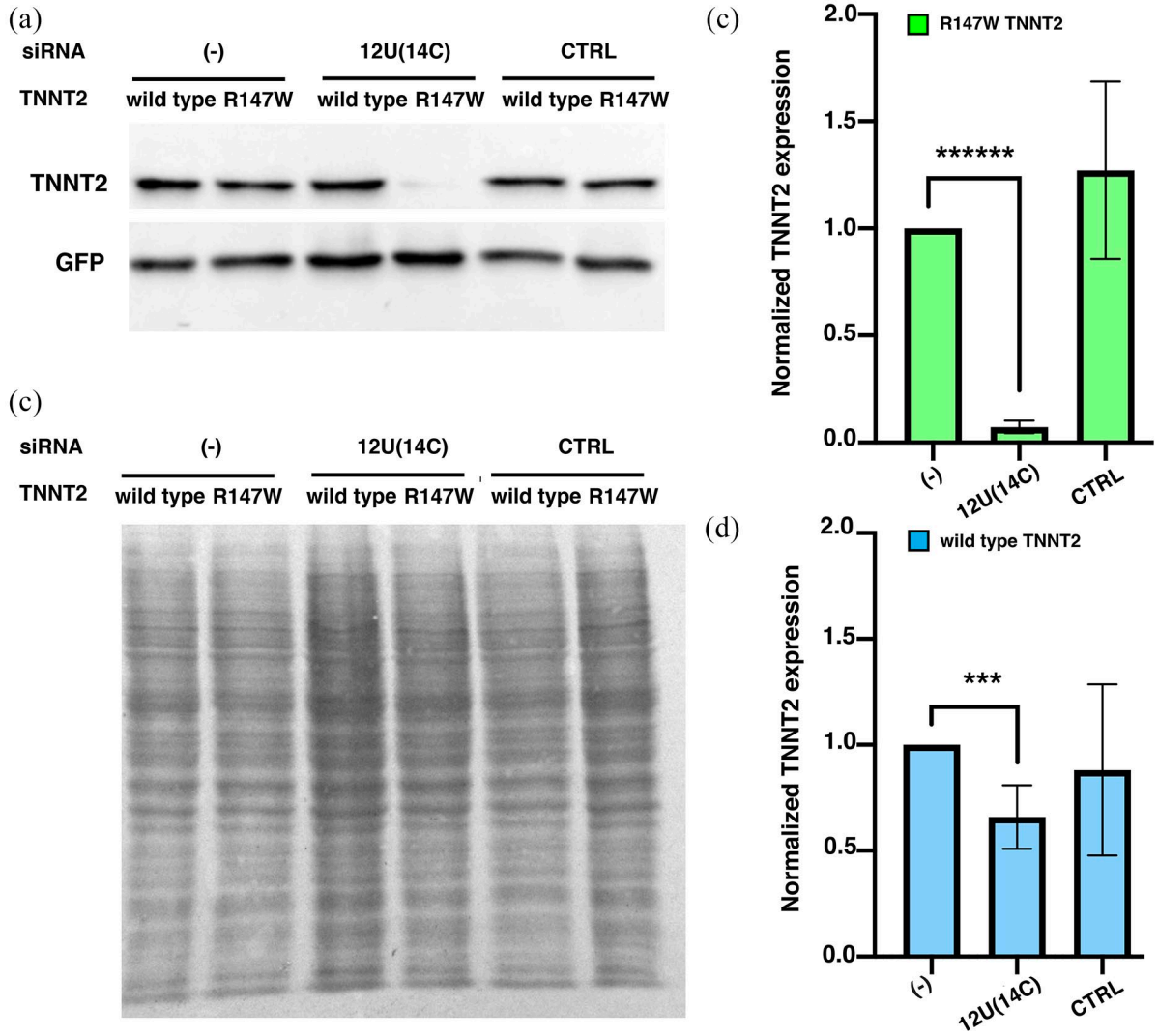

Figure 4. siRNA 12U (C14) silences the expression of R173W TNNT2 protein. (a) Representative western blot analysis of whole lysates of HEK293T cells co-transfected with expression plasmids coding for full-length wild-type or R173W TNNT2 and control (CTRL) or 12U (14C) siRNA. Protein extracts of cells transfected only with TNNT2 expression plasmids were used as control (–). Transfection efficiency was evaluated by co-transfection with a GFP expression plasmid. (b) Ponceau-red staining of nitrocellulose membrane following protein transfer, related to western blot in (a). (c) R173W TNNT2 relative expression in control cells (–) and in cells transfected with either siRNA 12U (14C) or control siRNA (CTRL). (d) Wild-type TNNT2 relative expression in control cells (–) and in cells transfected with either siRNA 12U (14C) or control siRNA (CTRL). Data are presented as mean value ± SD of three independent experiments (******$P < 0.000005$; ***$P < 0.0005$). (A color version of this figure is available in the online journal.)

the function and structure of the entire troponin complex, thus resulting in a dominant-negative effect.[30] Mutations in *TNNT2* are clustered in the central and C-terminal region, which are involved in protein–protein interactions with the tropomyosin and the other troponin complex subunits.[31]

Currently, the pharmacological intervention for HCM and DCM is mainly focused on symptoms relief and prevention of sudden cardiac death. In the last decades, several efforts have been done to develop therapeutic approaches for these patients, aimed to directly prevent the adverse effects of pathogenic mutations. Among them, the ASP-RNAi represents a powerful and promising strategy to counteract genetic defects, targeting mutant alleles and leading to their degradation with minimal suppression of the corresponding wild-type allele.[32] Significant therapeutic benefits have been demonstrated in iPSC-CM from patients and in mouse models of HCM, long-QT syndrome, and centronuclear myopathy treated with this approach.[19,33–35]

In the present work, we developed an ASP-RNAi methodology to target two mutations in *TNNT2*, the c.275 G > A (p.R92Q) and c.517 C > T (p.R173W) mutations, identified in patients affected by HCM and DCM, respectively. One of the most critical steps in ASP-RNAi is siRNA design. Numerous studies suggested that siRNAs carrying disease-linked mutation in the central position have the potential to confer the best allele discrimination.[25,27,36–38] Usually, siRNA duplexes are planned to have a sequence fully complementary to the mRNA target so that the endonuclease AGO2 of RISC complex is activated. Interestingly, AGO2 cleaves the mRNA target at the position corresponding to the center of the siRNA guide (between nucleotide positions 10 and 11).[39] Therefore, insert the disease-linked nucleotide substitution at the central position of siRNA is an important parameter to promote ASP-RNAi.

We thus designed siRNA duplexes of 19–21 nucleotides carrying the mutant nucleotide in the central region, in

positions ranging from 9 to 12 (p9 to p12) with respect to the first nucleotide of the sense strand for the 19-mer siRNAs and from p10 to p12 for the 21-mer siRNAs. Moreover, siRNAs duplexes present two UU nucleotide overhangs at their 3′-end that determine the asymmetry essential for the recognition and loading of siRNA guide into the RNAi machinery.[40]

We demonstrated that 19-mer siRNAs were able to inhibit the mutant allele in a more efficient way than the corresponding 21-mer siRNAs and that the ASP-RNAi was also largely affected by the position of mutant nucleotide in the siRNA sequence. We also found that siRNAs carrying the mutant nucleotide in a position between p9 and p12 are the most effective to silence the mutant TNNT2 alleles but induce low or no allele discrimination. These results agree with data from Trochet *et al.*[19], who demonstrated that positions 9 and 12 were the most selective in silencing the R465W mutant allele in DMN2, as well as by Takahashi *et al.*[26] and Ohnishi *et al.*[28] for ASP-RNAi of *ALK2*-R206H mutation and *PRNP*-D178N mutation, respectively.

It has been described that the type of nucleotide mismatch might influence the siRNA efficacy. In this context, Du and collaborators tested 20 siRNAs against 400 mismatched targets and demonstrated that different tolerance levels could be observed for the mismatches.[41] In particular, purine:purine or pyrimidine:pyrimidine base pairing between guide siRNA and mRNA are less tolerated, leading to the greatest level of discrimination.[23,27,42] This observation may explain the lower allele discrimination observed with the 19-mer siRNA targeting the c.275 G > A (p.R92Q) and the c.517 C > T (p.R173W) mutations in *TNNT2* where a change of a purine to a purine and a pyrimidine to a pyrimidine is present.

To enhance the ability of siRNAs to efficiently discriminate between mutant and wild-type alleles, a second mismatched nucleotide in the siRNA sequence was included. In detail, we focused on positions 2–8 at the 5′-end of guide strand of the siRNA, corresponding to the seed region, that plays a key role in recognition of the target mRNA by miRNA.[43] It has been suggested that the inclusion of a mismatch in this site can improve siRNA selectively and enhance the ASP-RNAi.[26,28,43–46] In fact, the addition of a nucleotide mismatch into the seed region induces one base unpairing between siRNA and mutant allele and two base unpairings between siRNA and wild-type allele, resulting in the reduction or in the loss of the wild-type allele recognition by the siRNA, while the targeting of the mutant allele is maintained.

We designed three siRNAs targeting the c.275 G > A and four siRNAs targeting the c.517 C > T mutation, carrying a mismatch into the seed region at different positions. As shown by the luciferase analysis, all the siRNAs carrying double mismatches improved the allele discrimination, although with different efficiency, suggesting that position and the choice of the base replacement are critical aspects to be evaluated.

It is worth noting that the sequences of the most efficient siRNAs, the 10A (13U) and the 12U (14C) targeting c.275 G > A (R92Q) and c.517 C > T (R173W) mutations, respectively, carry the target mutation and the additional mismatched nucleotide close one to each other and divided by one and two nucleotides, respectively. The analysis of protein expression confirmed the strong efficiency of siRNA 10A (13U) and 12U (14C) to repress translation of the mutant alleles with neither or minimal inhibition of the wild-type allele. To compare our results with those reported in the literature, we plotted the fold-difference expression of wild-type and mutant alleles obtained for *TNNT2*, *SOD1*, *COL6A1*, *ALK2*, and *PRNP* genes,[22,23,26,47] by means of ASP-RNAi, in function of the number of nucleotides (from 1 to 7) separating the double mismatch of the siRNA (Supplemental Figure S2). Although there is a large experimental variability and different model cell lines have been used, these data suggest that, in general, the double mismatched siRNAs differing by one or two nucleotides induce the strongest ASP-RNAi. Probably, this specific arrangement in the siRNA sequence, with the double mismatch occurring in a restricted region, results in the higher degree of destabilization of the wild-type mRNA–siRNA pairing.

In conclusion, our results show that optimal allele discrimination was obtained with the use of siRNAs carrying a double-base mismatch both for the R92Q and the R173W mutations. We are aware about the use of a non-muscle cell line, the HEK293T cells, that certainly represents a limit of this study since they do not express endogenous TNNT proteins and are not suitable for functional studies on cardiac contractility. Nevertheless, *in vitro* cell models are often used as validation platforms to test siRNA efficiency. We are confident that results obtained with the 10A (13U) and 12U (14C) siRNAs may easily be translated to other components of the contractile machinery of striated muscles and represent a starting point to design an ASP-RNAi therapy for HCM and DCM. Accordingly, ASP-RNAi therapy can be applied to virtually all mutations associated with human diseases with an autosomal dominant inheritance, including genes coding for the other components of the contractile machinery of striated muscles.[48,49]

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data, and review of the manuscript. L.M. conducted the experiments. F.G. supplied the critical equipment and expertise for luciferase assay. L.M., F.G., and D.R. wrote the manuscript. V.S., D.R., and E.P. supervised the study and contributed critical reading of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

REFERENCES

1. Braunwald E. Cardiomyopathies: an overview. *Circ Res* 2017;**121**:711–21
2. Morimoto S. Sarcomeric proteins and inherited cardiomyopathies. *Cardiovasc Res* 2008;**77**:659–66
3. Deo R, Albert CM. Epidemiology and genetics of sudden cardiac death. *Circulation* 2012;**125**:620–37
4. McKenna WJ, Maron BJ, Thiene G. Classification, epidemiology, and global burden of cardiomyopathies. *Circ Res* 2017;**121**:722–30
5. Richardson P, McKenna W, Bristow M, Maisch B, Mautner B, O'Connell J, Olsen E, Thiene G, Goodwin J, Gyarfas I, Martin I, Nordet P. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of Cardiomyopathies. *Circulation* 1996;**93**:841–2
6. Olivotto I, d'Amati G, Basso C, Van Rossum A, Patten M, Emdin M, Pinto Y, Tomberli B, Camici PG, Michels M. Defining phenotypes and disease progression in sarcomeric cardiomyopathies: contemporary role of clinical investigations. *Cardiovasc Res* 2015;**105**:409–23
7. Perry SV. Troponin T: genetics, properties and function. *J Muscle Res Cell Motil* 1998;**19**:575–602
8. Risi CM, Pepper I, Belknap B, Landim-Vieira M, White HD, Dryden K, Pinto JR, Chase PB, Galkin VE. The structure of the native cardiac thin filament at systolic Ca2+ levels. *Proc Natl Acad Sci USA* 2021;**118**:e2024288118
9. Bin W, Jin J-P. TNNT1, TNNT2, TNNT3: isoform genes, regulation, and structure-function relationships. *Gene* 2016;**582**:1–13
10. Hershberger RE, Parks SB, Kushner JD, Li D, Ludwigsen S, Jakobs P, Nauman D, Burgess D, Partain J, Litt M. Coding sequence mutations identified in MYH7, TNNT2, SCN5A, CSRP3, LBD3, and TCAP from 313 patients with familial or idiopathic dilated cardiomyopathy. *Clin Transl Sci* 2008;**1**:21–6
11. Tadros HJ, Life CS, Garcia G, Pirozzi E, Jones EG, Datta S, Parvatiyar MS, Chase PB, Allen HD, Kim JJ, Pinto JR, Landstrom AP. Meta-analysis of cardiomyopathy-associated variants in troponin genes identifies loci and intragenic hot spots that are associated with worse clinical outcomes. *J Mol Cell Cardiol* 2020;**142**:118–25
12. Morimoto S, Yanaga F, Minakami R, Ohtsuki I. Ca2+-sensitizing effects of the mutations at Ile-79 and Arg-92 of troponin T in hypertrophic cardiomyopathy. *Am J Physiol* 1998;**275**:C200–7
13. Alcalai R, Seidman JG, Seidman CE. Genetic basis of hypertrophic cardiomyopathy: from bench to the clinics. *J Cardiovasc Electrophysiol* 2008;**19**:104–10
14. Sun N, Yazawa M, Liu J, Han L, Sanchez-Freire V, Abilez OJ, Navarrete EG, Hu S, Wang L, Lee A, Pavlovic A, Lin S, Chen R, Hajjar RJ, Snyder MP, Dolmetsch RE, Butte MJ, Ashley EA, Longaker MT, Robbins RC, Wu JC. Patient-specific induced pluripotent stem cells as a model for familial dilated cardiomyopathy. *Sci Transl Med* 2012;**4**:130–47
15. Harada K, Potter JD. Familial hypertrophic cardiomyopathy mutations from different functional regions of troponin T result in different effects on the pH and Ca2+ sensitivity of cardiac muscle contraction. *J Biol Chem* 2004;**279**:14488–95
16. Hershberger RE, Pinto JR, Parks SB, Kushner JD, Li D, Ludwigsen S, Cowan J, Morales A, Parvatiyar MS, Potter JD. Clinical and functional characterization of TNNT2 mutations identified in patients with dilated cardiomyopathy. *Circ Cardiovasc Genet* 2009;**2**:306–13
17. Repetti GG, Toepfer CN, Seidman JG, Seidman CE. Novel therapies for prevention and early treatment of cardiomyopathies. *Circ Res* 2019;**124**:1536–50
18. Giorgio E, Lorenzati M, Rivetti di Val Cervo P, Brussino A, Cernigoj M, Della Sala E, Bartoletti Stella A, Ferrero M, Caiazzo M, Capellari S, Cortelli P, Conti L, Cattaneo E, Buffo A, Brusco A. Allele-specific silencing as treatment for gene duplication disorders: proof-of-principle in autosomal dominant leukodystrophy. *Brain* 2019;**142**:1905–20
19. Trochet D, Prudhon B, Beuvin M, Peccate C, Lorain S, Julien L, Benkhelifa-Ziyyat S, Rabai A, Mamchaoui K, Ferry A, Laporte J, Guicheney P, Vassilopoulos S, Bitoun M. Allele-specific silencing therapy for Dynamin 2-related dominant centronuclear myopathy. *EMBO Mol Med* 2018;**10**:239–53
20. Barbaro V, Nasti AA, Del Vecchio C, Ferrari S, Migliorati A, Raffa P, Lariccia V, Nespeca P, Biasolo M, Willoughby CE, Ponzin D, Palù G, Parolin C, Di Iorio E. Correction of mutant p63 in EEC syndrome using siRNA mediated allele-specific silencing restores defective stem cell function. *Stem Cells* 2016;**34**:1588–600
21. Drouet V, Ruiz M, Zala D, Feyeux M, Auregan G, Cambon K, Troquier L, Carpentier J, Aubert S, Merienne N, Bourgois-Rocha F, Hassig R, Rey M, Dufour N, Saudou F, Perrier AL, Hantraye P, Déglon N. Allele-specific silencing of mutant huntingtin in rodent brain and human stem cells. *PLoS ONE* 2014;**9**:e99341
22. Ohnishi Y, Tokunaga K, Kaneko K, Hohjoh H. Assessment of allele-specific gene silencing by RNA interference with mutant and wild-type reporter alleles. *J RNAi Gene Silencing* 2006;**2**:154–60
23. Schwarz DS, Ding H, Kennington L, Moore JT, Schelter J, Burchard J, Linsley PS, Aronin N, Xu Z, Zamore PD. Designing siRNA that distinguish between genes that differ by a single nucleotide. *PLoS Genet* 2006;**2**:e140
24. de Yñigo-Mojado L, Martín-Ruíz I, Sutherland JD. Efficient allele-specific targeting of LRRK2 R1441 mutations mediated by RNAi. *PLoS ONE* 2011;**6**:e21352
25. Sibley CR, Wood MJ. Identification of allele-specific RNAi effectors targeting genetic forms of Parkinson's disease. *PLoS ONE* 2011;**6**:e26194
26. Takahashi M, Katagiri T, Furuya H, Hohjoh H. Disease-causing allele-specific silencing against the ALK2 mutants, R206H and G356D, in fibrodysplasia ossificans progressiva. *Gene Ther* 2012;**19**:781–5
27. Huang H, Qiao R, Zhao D, Zhang T, Li Y, Yi F, Lai F, Hong J, Ding X, Yang Z, Zhang L, Du Q, Liang Z. Profiling of mismatch discrimination in RNAi enabled rational design of allele-specific siRNAs. *Nucleic Acids Res* 2009;**37**:7560–9
28. Ohnishi Y, Tamura Y, Yoshida M, Tokunaga K, Hohjoh H. Enhancement of allele discrimination by introduction of nucleotide mismatches into siRNA in allele-specific gene silencing by RNAi. *PLoS ONE* 2008;**3**:e2248
29. Hohjoh H. Allele-specific silencing by RNA interference. *Methods Mol Biol* 2010;**623**:67–79
30. Lopes LR, Elliott PM. A straight forward guide to the sarcomeric basis of cardiomyopathies. *Heart* 2014;**100**:1916–23
31. Marston SB. How do mutations in contractile proteins cause the primary familial cardiomyopathies? *J Cardiovasc Transl Res* 2011;**4**:245–55
32. Hohjoh H. Disease-causing allele-specific silencing by RNA interference. *Pharmaceuticals* 2013;**6**:522–35
33. Jiang J, Wakimoto H, Seidman JG, Seidman CE. Allele specific silencing of mutant Myh6 transcripts in mice suppresses hypertrophic cardiomyopathy. *Science* 2013;**342**:111–4
34. Bongianino R, Denegri M, Mazzanti A, Lodola F, Vollero A, Boncompagni S, Fasciano S, Rizzo G, Mangione D, Barbaro S, Di Fonso A, Napolitano C, Auricchio A, Protasi F, Priori SG. Allele-specific silencing of mutant mRNA rescues ultrastructural and arrhythmic phenotype in mice carriers of the R4496C mutation in the ryanodine receptor gene (RYR2). *Circ Res* 2017;**121**:525–36
35. Matsa E, Dixon JE, Medway C, Georgiou O, Patel MJ, Morgan K, Kemp PJ, Staniforth A, Mellor I, Denning C. Allele-specific RNA interference rescues the long-QT syndrome phenotype in human-induced pluripotency stem cell cardiomyocytes. *Eur Heart J* 2014;**35**:1078–87
36. Feng X, Zhao P, He Y, Zuo Z. Allele-specific silencing of Alzheimer's disease genes: the amyloid precursor protein genes with Swedish or London mutations. *Gene* 2006;**371**:68–74
37. Xia X, Zhou H, Huang Y, Xu Z. Allele-specific RNAi selectively silences mutant SOD1 and achieves significant therapeutic benefit in vivo. *Neurobiol Dis* 2006;**23**:578–86

38. Miller VM, Gouvion CM, Davidson BL, Paulson HL. Targeting Alzheimer's disease genes with RNA interference: an efficient strategy for silencing mutant alleles. *Nucleic Acids Res* 2004;**32**:661–8
39. Ameres SL, Martinez J, Schroeder R. Molecular basis for target RNA recognition and cleavage by human RISC. *Cell* 2007;**130**:101–12
40. Walton SP, Wu M, Gredell JA, Chan C. Designing highly active siRNAs for therapeutic applications. *FEBS J* 2010;**277**:4806–13
41. Du Q, Thonberg H, Wang J, Wahlestedt C, Liang Z. A systematic analysis of the silencing effects of an active siRNA at all single-nucleotide mismatched target sites. *Nucleic Acids Res* 2005;**33**:1671–7
42. Chiu YL, Rana TM. siRNA function in RNAi: a chemical modification analysis. *RNA* 2003;**9**:1034–48
43. Lai EC. Micro RNAs are complementary to 3'UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat Genet* 2002;**30**:363–4
44. Chipman LB, Pasquinelli AE. miRNA targeting: growing beyond the seed. *Trends Genet* 2019;**35**:215–22
45. Humphreys DT, Westman BJ, Martin DI, Preiss T. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. *Proc Natl Acad Sci USA* 2005;**102**:16961–6
46. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;**136**:215–33
47. Noguchi S, Ogawa M, Kawahara G, Malicdan MC, Nishino I. Allele-specific gene silencing of mutant mRNA restores cellular function in Ullrich congenital muscular dystrophy fibroblasts. *Mol Ther Nucleic Acids* 2014;**3**:e171
48. Dweck D, Sanchez-Gonzalez MA, Chang AN, Dulce RA, Badger CD, Koutnik AP, Ruiz EL, Griffin B, Liang J, Kabbaj M, Fincham FD, Hare JM, Overton JM, Pinto JR. Long term ablation of protein kinase A (PKA)-mediated cardiac troponin I phosphorylation leads to excitation-contraction uncoupling and diastolic dysfunction in a knock-in mouse model of hypertrophic cardiomyopathy. *J Biol Chem* 2014;**289**:23097–111
49. Martins AS, Parvatiyar MS, Feng HZ, Bos JM, Gonzalez-Martinez D, Vukmirovic M, Turna RS, Sanchez-Gonzalez MA, Badger CD, Zorio DAR, Singh RK, Wang Y, Jin JP, Ackerman MJ, Pinto JR. In vivo analysis of troponin C knock-in (A8V) mice: evidence that TNNC1 is a hypertrophic cardiomyopathy susceptibility gene. *Circ Cardiovasc Genet* 2015;**8**:653–64

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