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Original Article

ORIGINAL ARTICLE

Use of antisense oligonucleotides to correct the splicing error in ISCU myopathy patient cell lines

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Abstract

ISCU myopathy is an inherited disease that primarily affects individuals of northern Swedish descent who share a single point mutation in the fourth intron of the ISCU gene. The current study shows correction of specific phenotypes associated with disease following treatment with an antisense oligonucleotide (ASO) targeted to the site of the mutation. We have shown that ASO treatment diminished aberrant splicing and increased ISCU protein levels in both patient fibroblasts and patient myotubes in a concentration dependent fashion. Upon ASO treatment, levels of SDHB in patient myotubular cell lines increased to levels observed in control myotubular cell lines. Additionally, we have shown that both patient fibroblast and myotubular cell lines displayed an increase in complex II activity with a concomitant decrease in succinate levels in patient myotubular cell lines after ASO treatment. Mitochondrial and cytosolic aconitase activities increased significantly following ASO treatment in patient myotubes. The current study suggests that ASO treatment may serve as a viable approach to correcting ISCU myopathy in patients.

Introduction

Iron-sulphur clusters are found in essentially every living organism (1). These inorganic cofactors are used in a wide variety of proteins for many functions ranging from DNA replication and repair in the nucleus (2,3) to energy production in the mitochondria (1,4). Aside from possessing a high iron–sulphur cluster content, mitochondria are also a major site of iron–sulphur cluster biosynthesis. The biosynthesis of these cofactors involves numerous proteins, including the scaffold protein ISCU, the primary site of assembly of nascent iron–sulphur clusters (5). A complex of ISCU, cysteine desulfurase NFS1, its partner protein ISD11, ferrodoxin and frataxin are involved in the biosynthesis of iron-sulphur clusters. NFS1 and ISD11 are required for the production and donation of the sulphide moiety of iron–sulphur clusters. Ultimately, ISCU in complex with co-chaperones HSC20 and HSPA9 transfers the iron–sulphur cluster to recipient proteins (6).

In 1964, a report of individuals of northern Swedish descent with myopathy and poor physical performance since childhood was published (7). This work describing 14 patients from five families concluded that the condition was inherited and likely the result of a monohybrid recessive autosomal gene. In

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addition, nine of these patients were reported at some point as having dark urine due to the presence of myoglobin. Upon examination these patients demonstrated high levels of lactate and pyruvate in the blood following physical exertion as well as poor oxygen utilization. Subsequent studies demonstrated that activities of several iron-sulphur containing enzymes were deficient in these patients (8–11). The greater deficiencies were observed in succinate dehydrogenase (complex II) and aconitase and to a lesser extent in NADH dehydrogenase (complex I), cytochrome bc1 complex (complex III), and the Rieske protein (10).

In 2008, the mechanism of the disease was attributed to a point mutation in the 4th intron of the gene for ISCU (g.7044G→C) (12,13) that amplifies a polypyrimidine tract, thereby strengthening an otherwise weak splice acceptor site that permits inclusion of an aberrant exon in the processed transcript. The sequence including the weak polypyrimidine tract (tctttG) is in the 4th intron of the normal gene for ISCU and begins 401 bases after the termination of Exon 4. However, the mutation of the purine, guanine, to the pyrimidine, cytosine, in patients with ISCU myopathy extends this polypyrimidine tract by one base, creating the sequence tctttC. The longer polypyrimidine tract favours formation of a functional splice acceptor site, which leads to the formation of an aberrant exon that encodes 20 incorrect amino acids before terminating with a stop codon. The degree of mis-splicing is tissue specific; the highest incidence of mis-splicing occurs in skeletal muscle (14,15).

Antisense oligonucleotide (ASO) treatment is an emerging therapy with two drugs granted US Food and Drug Administration (FDA) approval, fomivirsen and mipomersen (marketed as Vitravene and Kynamro respectively). The FDA approved the first of these drugs, fomivirsen, as a first generation antisense drug with phosphorothioate linkages between the sugar bases of the oligonucleotide. The 21-mer sequence of fomivirsen is complementary to mRNA encoding proteins of the major immediate-early region of cytomegalovirus (CMV) and is used in the treatment of CMV retinitis. It is not complementary to any known mRNA sequences in humans (16).

Mipomersen is a 20-mer sequence complimentary to the coding region of human apolipoprotein B (apoB). It is a second generation ASO in that it features not only the phosphorothioate linkage but also a 2' O-methoxy-ethyl moiety on the ribose group. ApoB is a precursor of lipoproteins and a targeted strategy of lowering apoB has been used to diminish low-density lipoprotein cholesterol levels (17). The FDA approved the use of mipomersen in the treatment of familial hypercholesterolemia. Both of these drugs are used to inhibit protein synthesis by inducing RNase H1-mediated degradation of the transcript (18). To date, no ASO drugs have been approved by the FDA that suppress mis-splicing of disease-associated transcripts; this is the goal of the current study.

Spinal muscular atrophy (SMA) is an autosomal recessive genetic disease caused by loss of function of motor neuron gene (SMN1). Due to point mutation within its paralog SMN2, most of the SMN2 transcript excludes exon 7, leading to the expression of a truncated protein. Nusinersen, a uniformly modified second generation ASO to promote exon 7 inclusion, is currently being investigated in multiple phase 3 clinical trials. Early studies have provided encouraging evidence of improved motor neuron function for Nusinersen in SMA patients (19).

A previous study utilized a 25-mer antisense phosphorodiamidate morpholino oligonucleotide targeted to the mutation site in the ISCU gene to restore normal splicing in patient fibroblasts as indicated by an increase in mRNA of the normal transcript (20). Morpholino chemistry relies on a substitution of a six-membered morpholine ring for the normal five-membered sugar backbone of nucleic acids, which lowers binding affinity, necessitating use of a longer morpholino. The uncharged morpholine backbone is less likely to interact with proteins in a non-selective manner (21). The morpholino group also provides a favourable advantage in that the six-membered ring structure is not susceptible to degradation by endogenous cellular nucleases. To date the FDA has approved no drugs utilizing morpholine chemistry, although one morpholine-based drug, eteplirsen, is currently in clinical trials. This drug is being studied for treatment of Duchenne muscular dystrophy by causing the translation machinery to skip exon 51 (22).

The current study utilized ASOs targeted to the point mutation in the 4th intron of the gene for ISCU. Unlike other diseases utilizing ASO therapy, in which the mechanism is to prevent protein synthesis or to encourage exon skipping, the intended mechanism of the ASOs in the current study was to correct mis-splicing that leads to the inclusion of a pseudoexon by blocking access of splicing factors to the intronic mutation site. The efficacy of the drug was evaluated in primary patient fibroblast and myotubular cell lines in which we evaluated the integrity and activities of proteins that receive their iron-sulphur clusters from ISCU.

Results

More than 200 ASOs that bound to different regions of the ISCU transcript were synthesized using advanced technologies that increase resistance to nucleases and enhance tolerability (Ionis). Multiple ASOs that overlapped the mutation site restored normal levels of ISCU transcript and protein in cell lines derived from patients (data not shown). The ASO selected for further testing contained sequences that flanked the mutation, bound to the cytosine point mutation, rather than to the wild-type guanine, and overlapped the start of the aberrant exon 4A (Fig. 1B).

ASO treatment increased ISCU protein levels in both patient fibroblast and myotubular cell lines

Fibroblasts and myoblasts from patients had decreased ISCU protein levels relative to control fibroblasts (Fig. 2A) and myoblasts (Fig. 2B). Following transfection with 30 nM of the ASO, the ISCU levels returned to levels similar to those of the control fibroblasts (Fig. 2A). SDHB (data not shown) was undetectable in the patient fibroblasts.

Similar to the results seen in the patient fibroblasts, ASO treatment increased ISCU protein levels in patient myotubes. ISCU protein levels were similar in both heterozygous and control cell lines, consistent with the lack of phenotype observed in heterozygous individuals. Following transfection with 200 nM of the ASO, the ISCU levels of the patient myotubes returned to levels similar to those of the control myotubes (Fig. 2B). SDHB levels were lower in patient myotubes than in either control or heterozygous myotubes, but increased following ASO treatment (Fig. 2C).

A titration of the patient fibroblasts with different concentrations of the ASO showed that ISCU protein levels increased following treatment with 5-10 nM of the ASO and recovered to the levels of those in control fibroblasts following treatment with 25 nM of the ASO (Fig. 3A). No additional recovery was observed following treatment with the ASO above this concentration.

A titration of the patient myotubes with different concentrations of the ASO showed that ISCU protein levels increased following treatment with 50, 100, and 150 nM of the ASO and

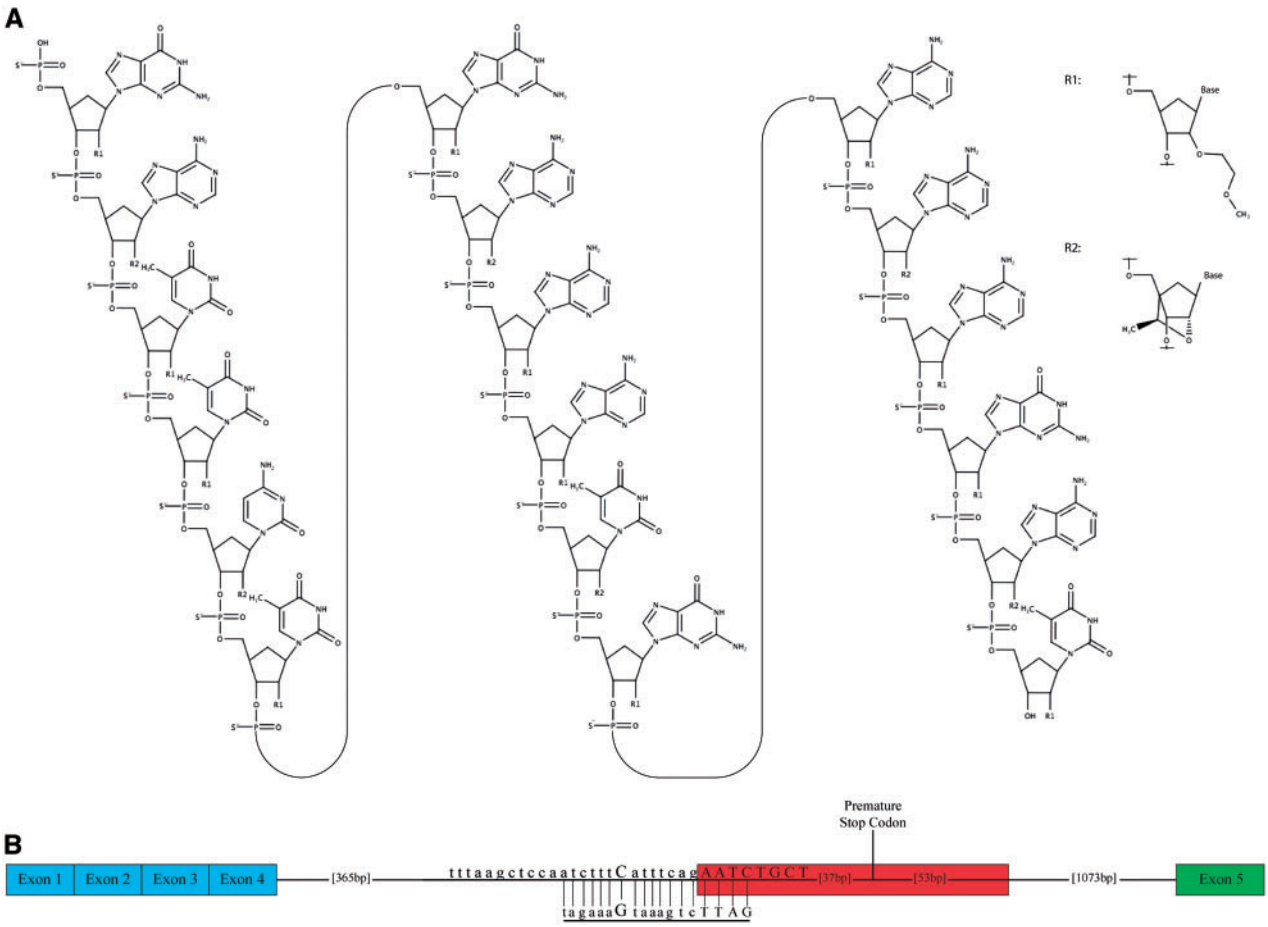

Figure 1. Depiction of the antisense oligonucleotide used in this study. (**A**). The structure of the antisense oligonucleotide is shown. The ASO features 2'-O-methoxyethyl (R1) and a constrained ethyl group (R2) modifications as shown. (**B**) A schematic of the site of binding on the gene. The 18mer oligonucleotide has the sequence of GATTCTGAAATGAAAGAT. The uppercase C on the schematic of the gene represents the location of the mutation in ISCU myopathy patients.

recovered to levels detected in the control myotubes following treatment with 200 nM of the ASO (Fig. 3B). Minimal additional recovery was observed following treatment with ASO above the 200 nM concentration.

ASO treatment increased expression of the correctly spliced transcript and suppressed the expression of the incorrectly spliced transcript in both patient fibroblast and myotubular cell lines

qRT-PCR analysis of patient fibroblast showed decreased levels of the region corresponding to Exon 2-3 prior to treatment with ASO. In addition, transcript levels of the region corresponding to Exon 4A were increased in patient fibroblast cell lines. Following ASO treatment, expression of the transcript sequence corresponding to Exon 2-3 increased, whereas expression of exon 4A decreased (Fig. 4A.) Similar to the results observed in the patient fibroblasts, ASO treatment of myoblasts decreased expression of aberrant exon 4A and increased expression of normal transcripts (Fig. 4B).

Complex II activity and succinate levels

Complex II activity measured by monitoring the change in OD at 600nm, corresponding to the reduction of DCPIP to $DCPIPH_2$, revealed that complex II activity in the patient fibroblasts was lower than in the control fibroblasts (Fig. 5A). Following treatment with 30 nM ASO, the Complex II activity of the patient fibroblasts was comparable to that of the control fibroblasts. The diminished complex II activity was more apparent in myotube cultures from patients; the complex II activity was about half the value of the control for the heterozygous myotubes and about one quarter the value of the control in the patient myotubes (Fig. 5B). Following treatment with 200 nM of ASO, complex II activity increased for the patient myotubes to values comparable with the complex II activity of the control myotubes. Comparing the different cell types, fibroblasts and myotubes, a more pronounced (~2 fold greater) deficiency was observed in the patient myotubes compared to the patient fibroblasts.

Succinate levels were assayed by an enzymatic reaction that results in the production of a colorimetric species with a maximal absorption of 450 nm. The concentration of succinate in the samples was calculated from a standard curve made from a serial dilution of a succinate solution of known concentration. In fibroblast cell lines, we observed minimal changes in the levels of succinate between control, patient, and ASO treated patient cell lines (Fig. 6A). In myotube cultures, succinate levels did not differ significantly between the heterozygous and control samples (Fig. 6B), whereas the concentration of succinate was significantly increased by ~2 fold in the patient myotube cultures. Following treatment with 200 nM of the ASO, the amount of

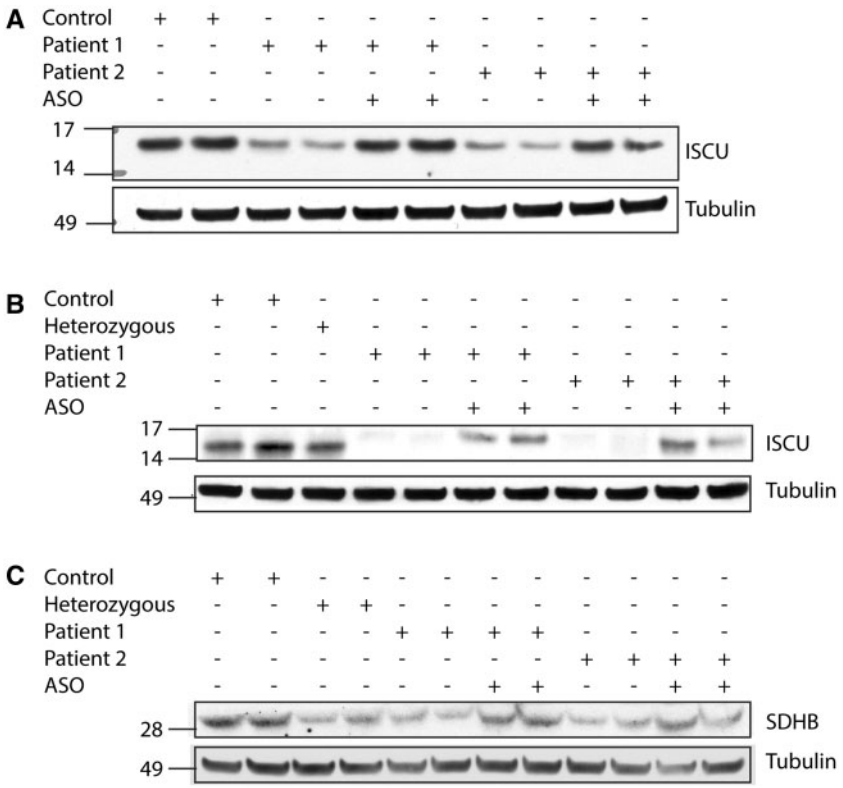

Figure 2. Western blots showed increased ISCU levels following ASO treatment. Western blots of fibroblasts (**A**) and myotubes (**B**) were probed for ISCU. Patient fibroblasts were treated with 30 nM ASO and patient myotubes were treated with 200 nM ASO. (**C**) Myotubes were also probed for SDHB. Tubulin is shown as a loading control for the experiments.

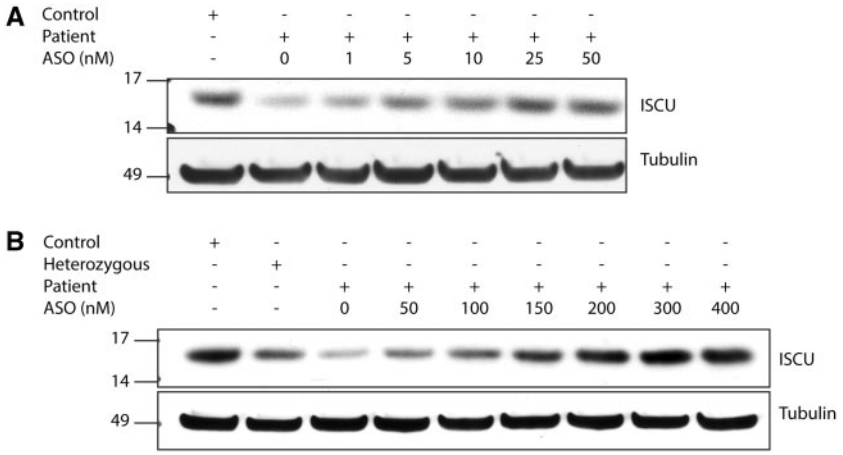

Figure 3. Concentration dependence of normalization of ISCU levels following the ASO treatment. Western blots of (**A**) fibroblasts and (**B**) myotubes were treated with various concentrations of ASO. Response to titration of 0, 1, 5, 10, 25, and 50 nM of ASO was assayed for the fibroblasts and response to a titration of 0, 50, 100, 150, 200, 300, and 400 nM was assayed for the myotubes. Tubulin was used as the loading control for the experiment.

succinate in the sample decreased to levels similar to those of the control and heterozygous samples.

Aconitase activity

In-gel aconitase activity stains showed little to no difference between patient and control fibroblasts (data not shown). In myotube cultures, levels of aconitase activity did not differ between the control and heterozygous cell lines. However, consistent with previous results (23), we observed lower activity of both mitochondrial and cytosolic aconitases for the patient myotube cultures compared to the heterozygous and control cell lines (Fig. 7). Aconitase activities returned to levels similar to those of both the heterozygous and control cell lines following treatment with 200 nM of the ASO.

Discussion

ISCU myopathy is a rare disease

ISCU myopathy is a rare disease that affects primarily skeletal muscle. The tissue specificity of the disease is intriguing since

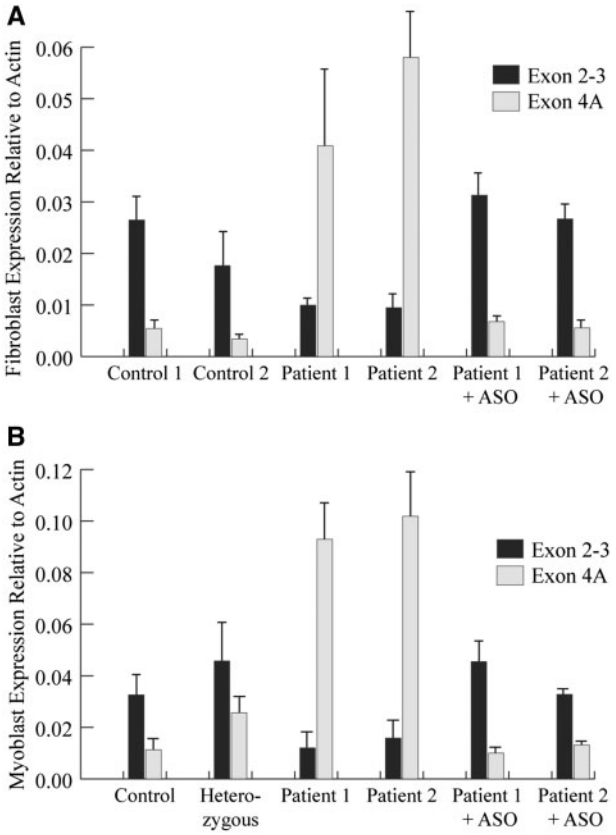

Figure 4. Restoration of normal splicing in fibroblasts and myotubes after ASO treatment. qRT-PCR detection of Exon 2-3 which represents the correctly spliced form of the transcript and Exon 4A which represents the mis-spliced of the transcript are compared in (**A**) fibroblasts and (**B**) myotubes ($n = 3$ for each sample).

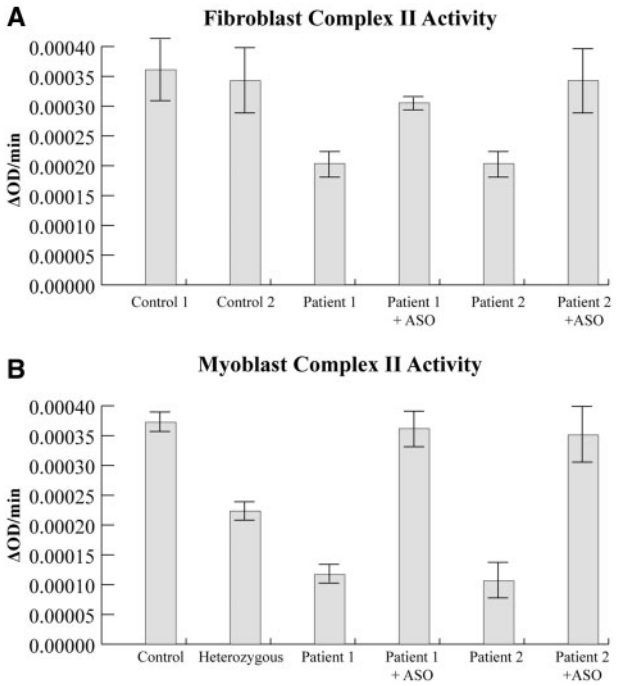

Figure 5. Restoration of Complex II activity in fibroblasts and myotubes after ASO treatment. The activity of complex II was assayed in the samples and the representative plots of (**A**) fibroblasts and (**B**) myotubes were made by dividing the change in absorbance over the same period of time for the samples. The period evaluated was within the linear range for the decrease in absorbance at 600nm corresponding to the reduction of DCPIP ($n = 4$ for each sample).

ISCU is expressed throughout the body. Specifically enhanced aberrant splicing in skeletal muscle may account for the fact that disease manifestations are primarily limited to the skeletal muscles (14,15,24). It has been suggested that high activity of the splicing factor, polypyrimidine tract binding protein 1 (PTBP1), may be involved in generating the tissue specificity (24). ISCU myopathy can be quite devastating for patients by severely limiting their ability to engage in physical activity; nevertheless, they often are able to cope with the disease by knowing their limitations and refraining from excessive exertion. These limitations are consistent with the observation that residual low levels of ISCU protein are observed in the western blots of patient skeletal muscle biopsies (23) and in patient fibroblasts and myotubes (Figs 2 and 3). Complete knockout of ISCU expression was previously shown to be embryonic lethal in mice (15).

Rare diseases are often not studied and treatments are not developed because only a small number of patients may benefit, and the disease rarity complicates assessment of treatment efficacy. The typical model for drug development includes a phase III clinical trial wherein a large cohort is given the drug and its efficacy and side effects are monitored, but ISCU myopathy is an ultra-rare disease for which treatment could not be assessed using a traditional approval process. The 1983 Orphan Drug Act has promulgated the development of several new treatments for rare diseases (25), but the process of Accelerated Approval has rarely been used for treatment of rare diseases (26). The largest hurdle for rare diseases to qualify for accelerated approval has been the fact that a defined endpoint biomarker does not exist in many cases (27).

In the case of ISCU myopathy many potential biomarkers could be used to assess the efficacy of ASO treatment for ISCU myopathy. Considerations should be made in testing these biomarkers, as some of these potential biomarkers such as activity tests are non-invasive but require specialized testing and additional expertise to interpret the results (8). Certainly, if patients are able to endure further physical activity following ASO treatment, their increased endurance could indicate that the drug is working well, and by normalizing to pre-treatment measurements, these tests could serve as clinical endpoints. Assessment of other biochemical biomarkers such as assaying the tissue for increases in ISCU protein could be more quantitative, but they would require an invasive biopsy and increased pain for the patients. To this end, a biomarker that is not as invasive as a muscle biopsy but is more quantitative than an activity test would be ideal. One potential biomarker, fibroblast growth factor 21 (FGF21) appears to be a biomarker of skeletal muscle health (28). Recent studies have shown that patients with ISCU myopathy have elevated levels of FGF21 (29), and serum assays could constitute a good test for the efficacy for ASO treatment in ISCU myopathy patients, as one would expect FGF21 serum levels to decrease following effective ASO treatment. In addition, levels of lactate and pyruvate, which are elevated in the patients, could be assayed as another potential biochemical marker of drug efficacy.

ISCU myopathy is a recessive autosomal condition in which heterozygous siblings of the patients are not affected, even though they express half the normal amount of ISCU in their muscles. The fact that ISCU levels are markedly diminished in asymptomatic patients demonstrates that full restoration of ISCU expression levels should not be necessary to successfully treat the disease.

Myotubes as a model of ISCU myopathy

The G→C point mutation that leads to ISCU myopathy is within the 4th intron of the human gene. The intronic sequence is specific to humans and likely represents the insertion of a retroposon into primates, and it has therefore been difficult to make an analogous mouse model. To make such a model would likely necessitate replacement of the entire murine *IscU* gene with the human *ISCU* sequence. However, the immortalized myoblasts were collected from the patients, and they recapitulate important aspects of the phenotype, producing a model that is free from genetic manipulation. Additionally, we have analysed fibroblasts collected from the patients, but the biochemical abnormalities were much milder in fibroblasts, as expected from the absence of other disease manifestations in patients.

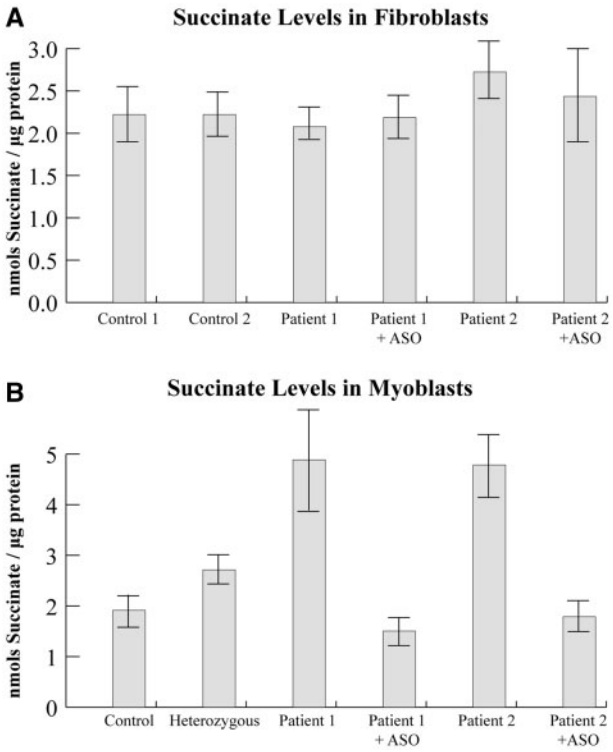

Figure 6. Normalization of succinate levels in fibroblasts and myotubes after ASO treatment. The concentration of succinate was measured in (**A**) fibroblasts and (**B**) myotubes. The concentration was calculated by absorbance at 450nm following an enzymatic reaction. Samples were normalized based on protein concentrations of the samples such that the same amount of protein was added for each measurement ($n = 4$ for each sample).

ISCU protein levels, correct splicing, and SDHB levels are restored following ASO treatment in fibroblast and myotubular cell lines

Regardless of whether we study patient-derived fibroblasts or myotubes, we observed that ISCU protein levels were lower in the cell lines from patients. Decreased expression of ISCU is consistent with previous findings and with the phenotype of the disease. For both cell types, treatment with the ASO restored ISCU protein levels to levels similar to those in both control and heterozygous cell lines. These experiments demonstrated that the ASO treatment is able to rescue the phenotype of lower ISCU protein levels regardless of cell type in a concentration dependent manner. qRT-PCR results demonstrated that ASO treatment increased the abundance of the transcript that includes Exons 2-3 which is representative of the correctly spliced transcript. Conversely the levels of transcripts that included exon 4A and are representative of the mis-spliced transcript decreased.

In the myotubular cell lines, we also observed lower protein levels of SDHB in the patient cell lines. Previous work has demonstrated that SDHB that is unable to obtain its iron-sulphur clusters undergoes rapid degradation (30). Our current findings of lower SDHB protein levels are consistent with this finding. It is likely that the impaired iron-sulphur cluster assembly machinery prevents the acquisition of the three essential iron-sulphur clusters on SDHB, and absence of the incorporation of the iron-sulphur clusters render the protein susceptible to degradation by the mitochondrial matrix protease, LonP (31). This notion is further strengthened by the fact that treatment with the ASO returned SDHB protein levels to normal. Deficiencies in SDHB in the myotubular cultures are consistent with previous findings on the disease, and the highest incidence of mis-splicing of ISCU occurs in skeletal muscle. This tissue is best modelled by the myotubular cell lines, so it was expected that even though the fibroblasts show detectably diminished ISCU protein levels, the pronounced biochemical phenotypes of decreased iron-sulphur cluster-containing protein activity were best recapitulated in the myotubular cell lines.

Complex II and succinate levels in fibroblast and myotubes cell lines

Complex II activity was slightly down in the fibroblasts, (factor of <2) whereas the effect was much more pronounced in

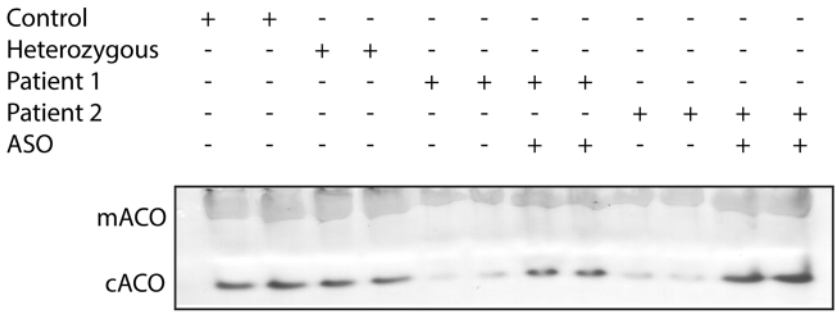

Figure 7. Restoration of aconitase activity following ASO treatment in In-gel aconitase assays of myotubes lysates. Myotubes were assayed for aconitase activity. Mitochondrial aconitase migrates at the higher position and cytosolic aconitase migrates to the lower position under these conditions.

myotubes (~4 fold decrease). In the heterozygous myotubes, a ~2 fold decrease in complex II activity was observed relative to the control myotubes. It is interesting that 50% retention of ISCU expression is sufficient to support the activity of important iron-sulphur enzymes, whereas further reduction of ISCU protein levels appears to cross a threshold that leads to loss of enzymatic activity and phenotypes. Following ASO treatment the patient myotubes had complex II activities that were comparable to the control myotubes. Decreased complex II activity has been previously noted in ISCU myopathy patients, so these results are consistent with previous studies (8). The increased complex II activity following ASO treatment is another example of reversal of the phenotype in these cell lines.

Deficiencies in complex II (succinate dehydrogenase) lead to the accumulation of succinate (32). In the patient fibroblasts where the loss of complex II activity was not as severe, we observed that levels of succinate were very similar between control, patient, and patient treated with ASO samples. In control and heterozygous myotubes we observed similar succinate levels, whereas we observed a ~2 fold increase in succinate levels in the patient myotubes relative to the control myotubes. Following ASO treatment, we observed that succinate levels decreased to normal. These findings correspond well with the measured complex II activities and are consistent with the conclusion that ASO treatment restored complex II activity in the patient myotubes.

Aconitase activity

Aconitase deficiency has also been noted in ISCU myopathy patients (8). As with SDHB protein levels and succinate levels, no change was seen in aconitase activity in the fibroblast cell lines (data not shown), consistent with the idea that the biochemical phenotype of the disease is not as penetrant in the fibroblast cell lines. Previously, deficiencies for both mitochondrial and cytosolic aconitases were observed in muscle tissue and myoblast cell lines (23). Similar results were observed in the patient myotubular cell lines in the current study. These deficiencies were corrected following ASO treatment. As with SDHB protein levels and complex II activity, restoration of aconitase activity represented another phenotype of the disease that was corrected by treatment with the ASO.

Implications of ASO treatment in other diseases

ASOs can be utilized in a number of ways to modulate disease conditions. Most of the antisense compounds in clinical studies are based on RNase H1 mediated degradation of the targeted transcript. In addition, ASOs can also modulate RNA processing events such as splicing, capping and polyadenylation, and can also block translation (18,33). Recently, it was demonstrated that ASOs can occlude upstream open reading frames (uORF) and increase protein expression from the main open-reading frame (34). The current study focused on using ASO treatment to prevent mis-splicing. Notably progress has been made utilizing ASOs in treating Duchenne muscular dystrophy (35). Duchenne muscular dystrophy is another condition that affects skeletal muscle; in Duchenne's, the ASO treatment is not targeted at preventing protein synthesis, but rather towards restoring a functional open-reading frame by preventing inclusion of mutation-bearing exons. There are several ongoing clinical trials utilizing ASO therapy to treat Duchenne muscular dystrophy (35). For ISCU myopathy, the goal is not to skip an exon but rather to exclude a portion of the intron from being included in the transcript. Using ASOs for this task is novel and may be an applicable therapy for other diseases caused by analogous mis-splicing events in which portions of an intron are retained in the mature mRNA. There are examples of intron inclusion in which an entire intron sequence is included such as limb girdle muscular dystrophy (36) and familial partial lipodystrophy type 2 (37). Both examples are the result of G→C point mutations (38) that alter the intronic splice donor site, reducing the ability of the splicing apparatus to correctly remove the intron. In this situation, use of an ASO would not be expected to enhance intron excision.

There are several examples in which a mutation generates a characteristic GT 5' splice donor site, or generates a characteristic AG 3' splice acceptor site that typically terminates an intron. These mutations lead to the inclusion of pseudoexons that arise from intronic sequence being included as aberrant exons. Examples of point mutations that generate cryptic splice donor sites include the DMD gene involved in Duchenne muscular dystrophy. A patient has been identified with an A→ G mutation in the intronic sequence between exons 25 and 26 creating the AG site (39). A similar A→G mutation found in the human oestrogen receptor, GHER, between exons 5 and 6 was found in breast cancer biopsy samples (40). In the gene CYBB, a G→T mutation between exons 5 and 6 leads to chronic granulomatous disease by creating a characteristic GT sequence leading to pseudoexon inclusion (41). A similar G→T mutation creates a GT donor site sequence in the gene PHEX, leading to the inclusion of a pseudoexon and gene dysfunction that leads to X-linked hypophosphatemia (42). A C→G mutation in the gene ornithine delta-aminotransferase, OAT, creates the characteristic GT sequence of a splice donor site leading to the inclusion of a pseudoexon, abnormal open reading frame and ornithine aminotransferase deficiency (43). More than 30 various rare disease presentations caused by pseudoexon formation have been identified and summarized elsewhere (44,45). In some instances, morpholino therapy has been used to correct splicing in fibroblast cell lines from affected patients, though the patients themselves were not treated (45). These examples indicate that numerous other diseases caused by abnormal exon inclusion due to intronic point mutations could be treated with ASOs designed to anneal to the mutation site, and the therapy tested in this paper for ISCU myopathy could therefore have broad implications in developing a new type of precision treatment that would be applicable to a growing subset of rare diseases.

The current study illustrates that specific phenotypes associated with ISCU myopathy can be corrected following treatment with an ASO targeted to the site of the mutation. We have shown that ASO treatment increases ISCU protein levels in both patient fibroblasts and patient myotubes. Furthermore, this increase occurs in a concentration-dependent fashion. We have shown that ASO treatment increases the abundance of the correctly spliced form of the transcript in patient fibroblasts and patient myotubes. We have also shown that specific biochemical manifestations of the disease are also corrected by ASO treatment. Specifically, we have shown that SDHB levels in patient myotubes cell lines increase to levels observed in control myotubular cell lines following ASO treatment. Additionally, we have shown that both patient fibroblast and myotubes cells lines display an increase in complex II activity following ASO treatment with a greater effect observed in the patient myotubular cell lines. Correspondingly, we observed a decrease in succinate levels in patient myotubes cell lines following ASO treatment. Finally, we observed an increase in aconitase activity

following ASO treatment in patient myotubes cell lines. Taken together these experiments suggest that ASO treatment would likely be an effective therapy for treating ISCU myopathy.

Materials and Methods

Primary cultures

Primary fibroblast and myoblasts of control individuals, heterozygous individuals, and ISCU myopathy patients were previously collected (23). Fibroblasts were cultured in DMEM medium containing 5 mM glucose and 1 mM sodium pyruvate (ThermoFisher) and supplemented with 10% foetal bovine serum (CellGro) 1% Antibiotic-Antimycotic (ThermoFisher). Prior to differentiation, primary myoblasts were cultured in SkBM-2 medium supplemented with human epidermal growth factor, dexamethasone, l-glutamine, foetal bovine serum and gentamicin/amphotericin-B as directed by the manufacturer (Lonza). For differentiation, myoblasts were cultured in DMEM medium (5 mM glucose, 1 mM Sodium pyruvate) supplemented with 2% heat-inactivated horse serum, 1% Antibiotic-Antimycotic (both from ThermoFisher) and 0.4 μg/ml dexamethasone (Sigma-Aldrich) for 4 days. Following differentiation, myotubes were cultured in DMEM medium (5 mM glucose, 1 mM Sodium pyruvate) supplemented with 10% FBS, 1% Antibiotic-Antimycotic and 0.4 μg/ml dexamethasone until cells were collected. All cells were grown in a low oxygen (5% O_2) incubator.

Antisense oligonucleotide treatment

Several ASOs targeted to the site of the mutation were tested for efficacy in relieving the phenotype of ISCU myopathy in fibroblast and myotube cell lines. Ionis Pharmaceuticals Inc. oligonucleotide 676672 with the antisense sequence GATTCTGAAATGAAAGAT containing chemical modifications to the ribose indicated in Fig. 1A was ultimately selected for further testing. For ASO treatment, fibroblasts in culture were rinsed with PBS and cells were incubated in Opti-MEM reduced serum medium (ThermoFisher scientific) supplemented with 5uL/ml Lipofectamine 2000 (ThermoFisher) and 30 nM ASO (Ionis Pharmaceuticals) unless otherwise noted; differentiated myotubes were also incubated in Opti-MEM reduced serum medium with 5uL/ml Lipofectamine 2000 and 200 nM ASO unless otherwise noted. Cells were incubated in this suspension for 4 h at 37°C, 5% O_2. Following these incubations, cells were returned to normal growth medium for 3 days before harvesting.

Western blot

Samples for western blot were collected by rinsing the cultured cell lines with PBS and then treating with trypsin (Cellgro) until the cells detached. Cells were then pelleted at 500 x g for 5 min and rinsed 3 times with PBS. Pellets were lysed with lysis buffer (10 mM Tris, 3 mM citrate, 150 mM NaCl, 2 mM $MgCl_2$, 1% Triton, and protease inhibitor) and the supernatant following centrifugation at 20,000x g was collected. The protein concentration was determined by the BCA method (ThermoFisher) and samples were prepared with NuPAGE LDS sample buffer and NuPAGE Sample reducing buffer (ThermoFisher). Samples were incubated at 95°C for 10min and then separated on NuPAGE Novex 4-12% Bis-Tris gels at 150V for 1h with MES SDS running buffer (Invitrogen). Protein was transferred to 0.2μm nitrocellulose membranes (Amersham) in Tris-glycine buffer (Invitrogen) with 15% methanol (Macron Fine Chemicals) at 120V for 1.5h at 4°C. Membranes were blocked with blotting grade blocker (Bio-Rad) for 1h and incubated with antibodies for ISCU described previously (46) and succinate dehydrogenase subunit B (SDHB) (Abcam) overnight at 4°C. Membranes were incubated with secondary antibodies (Sigma) and ECL substrate (ThermoFisher) before imaging on autoradiography film (Denville Scientific Inc.).

RNA preparation and qRT-PCR analysis

qRT-PCR was performed as described previously (23), with the following modifications: RNA from control and patient fibroblasts and control, heterozygous, and patient myotubes was collected using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), the RNA was reverse transcribed. qRT-PCR was performed using Fast SYBR® Green Master Mix and the primers listed in Table 1 of (23). Relative transcript abundance was calculated using the $2^{-\Delta\Delta CT}$ method (47), with Actin (forward GTCATTTCAGGTGAGAGG CTGC, reverse CAATGAAGTCCAGGGCCTTGTT) as the internal control.

Aconitase activity assay

Mitochondrial and cytosolic aconitase activities were assessed by in-gel aconitase assays performed as described previously (5). Cells were lysed as above and the following added: 25 mM Tris-Cl°H 8.0, 10% glycerol, and 0.025% bromophenol blue (final concentrations). Samples were loaded on non-denaturing gels with a separating gel composed of 8% acrylamide, 132 mM Tris base, 132 mM borate, 3.6 mM citrate, and a stacking gel composed of 4% acrylamide, 67 mM Tris base, 67 mM borate, 3.6 mM citrate. Samples were initially separated through the stacking gel at 120V for 20 min and then through the separating gel at 170V for 2.5 h with 25 mM Tris pH 8.3, 192 mM glycine, and 3.6 mM citrate running buffer. Aconitase activities were assayed by incubating the gel in 100 mM Tris (pH 8.0), 1 mM NADP, 2.5 mM cis-aconitic acid, 5 mM MgCl2, 1.2 mM MTT, 0.3 mM phenazine methosulfate, and 5 U/ml isocitrate dehydrogenase at 37°C in the dark.

Complex II activity assay and succinate concentration measurements

To measure complex II activity, unlysed samples were adjusted to protein concentration parameters specified in the enclosed protocol for the Complex II Enzyme Activity Microplate Assay Kit (Abcam). These samples were then lysed using the provided lysis buffer and the protein concentration was measured again using the BCA method to ensure equal loading amongst samples. The assay relies on the decrease in absorbance at 600nm that occurs upon the reduction of dichlorophenolindophenol (DCPIP) to yield a colourless product. Per the instructions, the ΔOD was normalized to time within the linear range of absorbance decay for the samples. Succinate levels were measured following the instructions provided with the Succinate Colorimetric Assay Kit (Sigma-Aldrich).

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Conflict of Interest statement. B.P.M, S.G, S.M.F. work at Ionis Pharmaceuticals. No conflict of interest exists for the other authors.

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