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Exploring Splicing-Switching Molecules For Seckel Syndrome Therapy

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ABSTRACT

The c.2101 A > G synonymous change (p.G674G) in the gene for ATR, a key player in the DNA-damage response, has been the first identified genetic cause of Seckel Syndrome (SS), an orphan disease characterized by growth and mental retardation. This mutation mainly causes exon 9 skipping, through an ill-defined mechanism.
Through *ATR* minigene expression studies, we demonstrated that the detrimental effect of this mutation (6 ± 1% of correct transcripts only) depends on the poor exon 9 definition (47 ± 4% in the ATR^{wt} context), because the change was ineffective when the weak 5′ or the 3′ splice sites (ss) were strengthened (scores from 0.54 to 1) by mutagenesis. Interestingly, the exonic c.2101 A nucleotide is conserved across species, and the SS-causing mutation is predicted to concurrently strengthen a Splicing Silencer (ESS) and weaken a Splicing Enhancer (ESE). Consistently, the artificial c.2101 A > C change, predicted to weaken the ESE only, moderately impaired exon inclusion (28 ± 7% of correct transcripts). The observation that an antisense oligonucleotide (AON^{ATR}) targeting the c.2101 A position recovers exon inclusion in the mutated context supports a major role of the underlying ESS.
A U1snRNA variant ($U1^{ATR}$) designed to perfectly base-pair the weak 5'ss, rescued exon inclusion (63 ± 3%) in the ATR^{SS}-allele. Most importantly, upon lentivirus-mediated delivery, the $U1^{ATR}$ partially rescued ATR mRNA splicing (from ~19% to ~54%) and protein (from negligible to ~6%) in embryonic fibroblasts derived from humanized ATR^{SS} mice.
Altogether these data elucidate the molecular mechanisms of the *ATR* c.2101 A > G mutation and identify two potential complementary RNA-based therapies for Seckel syndrome.

1. Introduction

Seckel syndrome (SS) is an extremely rare autosomal recessive disorder characterized by intrauterine grow retardation, dwarfism, microcephaly with mental retardation, and a "bird-headed" facial appearance [1]. So far, there is no cure and the molecular mechanisms are poorly known.

The first genetic defect found to be associated to SS (SS-type 1; OMIM 210600) has been the c. 2101 A > G synonymous (p.G674G) mutation in the gene encoding for the ataxia-telangiectasia and RAD3-related protein (ATR) [2], a phosphatidylinositol 3-kinase like kinase playing a key role in the DNA damage response [3–5]. Studies in patients' cells and *in vitro* [2] have shown that this mutation mainly causes exon 9 skipping and synthesis of a shorter and frame-shifted mRNA form. Trace levels of correct transcripts are also present, which explains a very severe but not lethal phenotype that is expected from a null-condition [6].

The c.2101 A > G change has been used as model to investigate the role of ATR protein, and a mouse model harboring the humanized *ATR* gene, and recapitulating the SS-1 features, has been developed [7]. Notwithstanding, the molecular mechanisms through which this nucleotide change induces exon 9 skipping have not been elucidated. Despite this splicing mutation represents an ideal model to explore RNA-based correction approaches [8,9] that are attracting great attention for many other human genetic diseases, no attempts have been made so far. Indeed, depending on the regulatory elements involved, the skipped exon could be rescued by masking negative elements through antisense molecules (U7snRNA or oligonucleotides) [10–12] or by increasing exon definition through variants of the U1 small nuclear RNA (U1snRNA) [13–25], the component of the ribonucleoprotein U1snRNP driving the recognition of the 5′ splice site (5'ss) in the earliest splicing steps [26].

In the present study, through expression studies with *ATR* minigenes we demonstrated that the c.2101 A > G synonymous change promotes the skipping of the very weak exon 9 mainly by strengthening an Exonic Splicing Silencer (ESS). This finding prompted us to develop two complementary correction approaches based on an antisense

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oligonucleotide masking the ESS or a modified U1snRNA with increased complementarity to the weak authentic exon 9 5'ss, which were able to remarkably rescue splicing. The U1snRNA was also challenged in Mouse Embryonic Fibroblasts (MEF) from the humanized SS-1 mouse, and resulted in partial rescue of ATR mRNA and protein levels.

2. Materials and Methods

2.1. Creation of vectors

2.1.1. ATR minigenes

To create the pATR^{wt} construct, the 1561-bp genomic fragment spanning *ATR* intron 8 (from position −434) trough intron 10 (until position +416)(Fig. 1a) was amplified from genomic DNA of a normal subject using high-fidelity PfuI DNA-Polymerase (Transgenomic, Glasgow, UK) with primers $^{5'}$TGACCATATGTGCACATCTTCACCTCTATT CTG$^{3'}$ (forward) and $^{5'}$TACGCATATGTGGAAAGTGGCCAAGAAGAT$^{3'}$ (reverse), and cloned in the pTB expression vector by exploiting the *NdeI* restriction site included in the primers (underlined). The mutant constructs pATR^{SS}, pATR$^{SS5'ss+}$, pATR$^{SS3'ss+}$ and pATR^{2101C} were generated by site-directed mutagenesis (QuickChange II XL Site directed Mutagenesis Kit; Agilent Technologies) (primers: pATR^{SS}, $^{5'}$CTAGTT GTGTTAGTGGGTTTTTTATCTTATTG$^{3'}$ and $^{5'}$CAATAAGATAAAAAACCCAC TAACACAACTAG$^{3'}$; pATR$^{SS5'ss+}$, $^{5'}$CCCAAGATT CTTAGGTATGTACTAA$^{3'}$ and $^{5'}$TTAGTACATACCTAAGAATCTTGGG$^{3'}$; pATR$^{SS3'ss+}$, $^{5'}$CTTAATTTTT TCAGGACCACAGGCACAATC$^{3'}$and $^{5'}$GATTGTGCCTGTGGTCCTGAAAA AATTAAG$^{3'}$; pATR^{2101C}, $^{5'}$CTAGTTGTGTTAGTGGCTTTTTTATCTTATTG$^{3'}$ and $^{5'}$CAATA AGATAAAAAAGCCACTAACACAACTAG$^{3'}$. All minigenes were validated by sequencing.

To create the pU1^{ATR} expression vector, the *Bgl*II-*Bcl*I fragment of the pU1wt was replaced using oligonucleotides $^{5'}$GATCTGATACATACATAG CAGGGGAGATACCAT$^{3'}$ and $^{5'}$GATCATGGT ATCTCCCCTGCTATGTATGT ATCA$^{3'}$ as previously described [14].

2.1.2. Lentiviral vector

For the creation of the U1^{ATR}-expressing lentivirus vector, the coding sequence for the U1^{ATR} was cloned in a lentiviral plasmid backbone (pLV- PGK-EGFP, Cyagen Biosciences) using the *BamHI* site. The pLV-U1^{ATR} lentiviral plasmid together with psPAX2 packaging and pMD2.G envelope plasmids were combined at a ratio of 3:2:1 respectively, and then used to transfect 293T packaging cells using calcium phosphate method. Twenty-four hours post transfection the medium was replaced with a new medium. The virus supernatants were collected at 48 and 72 h post transfection and filtered through a 0.45 μm filter.

2.2. Antisense oligonucleotide (AON)

The $^{5'}$UAAGAUAAAAAACCCACUAACACAA$^{3'}$ antisense oligoribo nucleotide (AON^{ATR}) was designed to cover the c.2101 position and in the light of the secondary structures predicted by the Sfold program

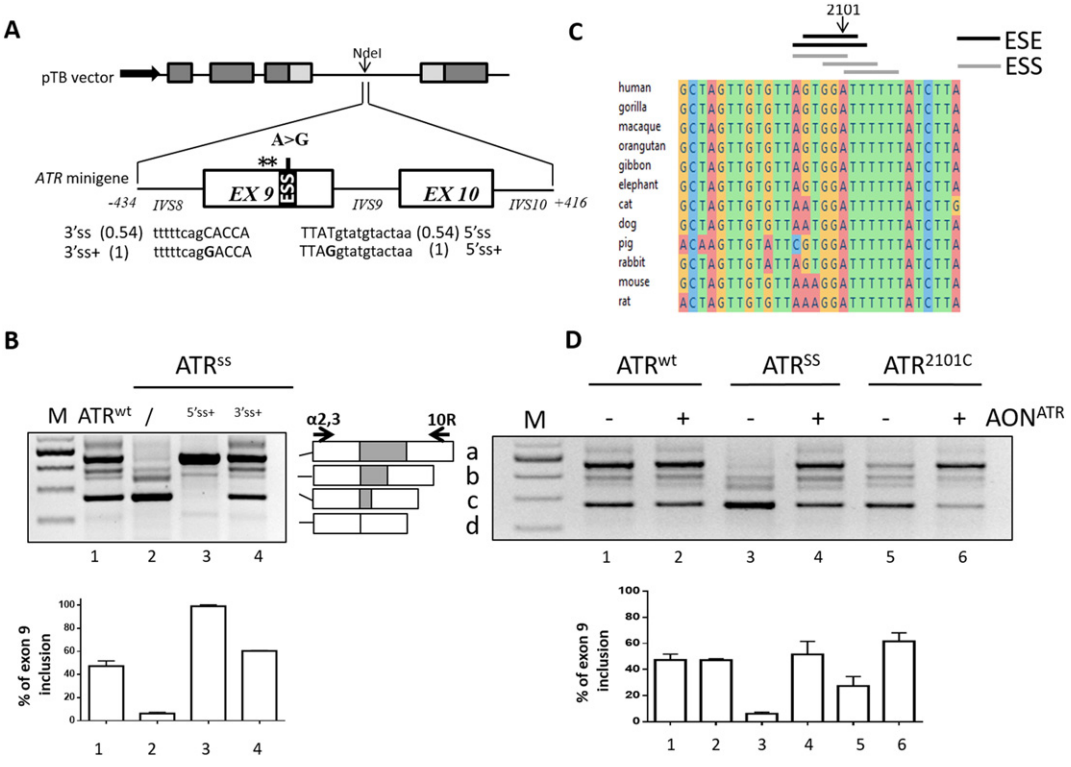

Fig. 1. Splicing features of the human *ATR* exon 9 context. A) Schematic representation of the *ATR* genomic sequence cloned as minigene in the pTB plasmid. Exonic and intronic sequences are represented by boxes and lines, respectively. Exons of the globin and fibronectin genes are indicated by grey and light grey boxes, respectively. The position of the c.2101 A > G (A > G) mutation and of cryptic 5'ss (asterics) are indicated above the *ATR* minigene. The black box represents the exonic splicing silencer (ESS) strengthened by the mutation. The intronic (lower cases) and exonic (upper cases) sequences and the scores (within parenthesis) of the 3'ss and 5'ss, either authentic or improved (+) by mutagenesis (nucleotides in bold), are reported below the scheme. B) Evaluation of ATR alternative splicing patterns in HEK293 cells transiently transfected with the indicated pATR constructs. The schematic representation of the normal (a) and aberrant (b-d) transcripts, and of primers used for the RT-PCR (arrows), is reported in the central panel. PCR amplified products were separated on 2% agarose gel. M, 100 bp molecular weight marker. Histograms indicate the relative percentage of correctly spliced transcripts upon densitometric analysis of bands, which is expressed as mean ± standard deviation (SD) from at least three independent experiments. C) Multiple sequence alignment of the region surrounding the c.2101 A position in various species. The position of the nucleotide under investigation, as well as the position of predicted ESE and ESS elements (dark and grey lines, respectively), are indicated above. D) Evaluation of ATR alternative splicing patterns in HEK293 cells transiently transfected with either the pATR minigenes alone (−) or in combination with 200 nM of AON^{ATR} (+). The schematic representation of transcripts and of primers used for the RT-PCR as well as the electrophoretic conditions and the densitometric analysis is detailed as in panel B.

(http://sfold.wadsworth.org/cgi-bin/index.pl). The AON^{ATR} was chemically synthesized to contain 2′-O-methyl modified RNA and a full-length phosphorothioate backbone [25].

2.3. Expression of ATR minigenes in mammalian cells and mRNA studies

Human Embryonic Kidney (HEK293) cells were cultured and transiently transfected with Lipofectamine 2000 (ThermoFisher SCIENTIFIC, Carlsbad, CA, USA) in 12-well plate as previously described [15]. pATR minigenes (1.5 μg) were transfected either alone, with AON^{ATR} or with 1.5× molar excess of pU1 vectors. Total RNA extraction was performed 24 h post-transfection using TRIreagent (ThermoFisher SCIENTIFIC) and reverse transcribed using the M-MLV (ThermoFisher SCIENTIFIC). The α-2,3 ($^{5'}$CAACTTCAAGCTCCTAAGCCACTGC$^{3'}$) and 10R ($^{5'}$GTCCACATGTCCGTGTTCAG$^{3'}$) primers were used for the following PCR that was run for 40 cycles at the following conditions: 95 °C for 30 s, 56 °C for 30 s, 72 °C for 40 s. Densitometric analysis of bands upon electrophoresis on 2% agarose gel was performed using ImageJ software.

2.4. Infection of immortalized MEF cells and ATR mRNA/protein studies

Seckel mouse embryonic fibroblast (MEF $ATR^{S/S}$) cells were incubated with the filtered virus supernatant supplemented with polybrene (8 μg/ml) at least for 3 h at 37 °C in a humidified incubator. Afterwards the medium was replaced by fresh medium. The RNA was isolated and reverse transcribed with Random hexamers as above mentioned and PCR amplified with primers 8F ($^{5'}$CCATTCTGATGATGGCTGTTT$^{3'}$) and 10R (see above) for 40 cycles at the following conditions: 95 °C for 30 s, 56 °C for 30 s, 72 °C for 40 s. Immortalized wild-type MEF, not harboring the human ATR cassette, were used as control to assess the impact on mouse ATR splicing though primers m8F ($^{5'}$CTACTAAC CTTCTGCCATTAAGCCAG$^{3'}$) and m10R ($^{5'}$AACATGCCGTGAAGAGTACA GAC$^{3'}$).

For the ATR protein analysis, cells were washed twice with ice-cold PBS and then directly lysed with 2× Laemmli buffer (120 mM Tris-HCl pH 6.8, 4% SDS and 20% Glycerol) supplemented with protease and phosphatase inhibitors. After sonication, the lysate was cleared by centrifugation and the supernatant was recovered. The protein concentration was quantified using optical absorbance at 280 nm. 25 μg of protein was boiled with β-mercaptoethanol (5% *v*/v) at 95 °C for 10 min. Samples were then separated on 4–15% gradient precast TGXTM polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA, USA) and then were blotted onto nitrocellulose membrane using Trans-Blot® TurboTM transfer system (25 V, 1 A, 30 min). Following 1 h blocking in TBST containing 5% BSA, the blot was incubated with primary antibody against ATR (N-19, 1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX, USA) for overnight at 4 °C. Secondary peroxidase-coupled antibody (rabbit anti-goat, Sigma-Aldrich, 1:5000 dilution) was incubated with the blot at room temperature for 1 h. ECL-based chemiluminescence (Bio-Rad Laboratories) was detected on BioRad ChemiDoc system and the image was processed using Image Lab 4.0 (Bio-Rad Laboratories).

2.5. Computational analysis

Bioinformatic prediction of splice sites and/or splicing regulatory elements was conducted by using the http://fruitfly.org/seqtools/splice.html and the Human Splicing Finder (http://www.umd.be/HSF3/) online softwares.

3. Results and Discussion

The knowledge of molecular mechanisms regulating the splicing process, and involving the interplay between positive and negative regulatory elements located within exons and/or introns, offers the opportunity to design novel therapeutic approaches for splicing mutations, a relevant cause of human disease [27–29]. It is worth noting that RNA-based correction strategies have the advantage of maintaining the gene regulation only in the physiologic tissue and of exploiting small therapeutic cassettes that can be delivered by any viral vector suitable for gene therapy.

In this paper, to explore a correction approach for Seckel syndrome, so far never attempted, we chose as model the Seckel Syndrome-1 causing *ATR* c.2101 A > G synonymous mutation that mainly promotes exon 9 skipping, and makes it an ideal target for splicing-switching molecules. The study took advantage of the expression of *ATR* minigenes, which can be easily manipulated for mechanistic investigations, and of *ex-vivo* models represented by embryonic fibroblasts from a humanized SS-1 mouse model, which recapitulates the SS-1 phenotype [7].

3.1. The c.2101 A > G mutation strengthens an Exonic Splicing Silencer in the intrinsically poorly defined exon 9

To dissect the molecular mechanism of the c.2101 A > G change we expressed the *ATR* minigene spanning exon 8 through 10 (Fig. 1a), either wild-type ($pATR^{wt}$) or mutated ($pATR^{SS}$), in the pTB backbone. The analysis of splicing patterns in transfected HEK293 cells recapitulated those previously described in the affected patients [2]. In particular, this mutation not only promoted exon 9 skipping (68 ± 2% of transcripts)(Fig. 1b) but also the usage of two exonic 5'ss cryptic sites at positions c.2093 and c.2075 (Fig. 1a, asterisks), in all cases producing frame-shifted transcripts. Trace levels (6 ± 1%) of the correctly spliced transcripts were also detected, which exclude a null ATR condition that would be lethal [6].

These data validated our experimental system and recapitulated the ATR processing in patients, thus providing us with a model for mechanistic studies. The occurrence of exon-skipping even in the wild-type context (29 ± 4% of transcripts) indicated the poor definition of exon 9, in part attributable to the remarkable divergence of the 5'ss (score 0.54) and 3'ss (0.54) sequences from the consensus ones. We therefore investigated the splicing patterns in an artificial context in which exon 9 definition was improved by increasing the 5'ss ($pATR^{SS5'ss+}$) or the 3'ss ($pATR^{SS3'ss+}$) scores (Fig. 1a). Noticeably, in both cases the impact of the c.2101 A > G mutation was virtually abolished (Fig. 1b), with the ameliorated 5'ss context associated only with correct transcripts.

Comparative analysis showed that the c.2101 A nucleotide is conserved across species, and bio-informatic analysis of splicing regulatory elements predicted that the A to G substitution strengthens an Exonic Splicing Silencer (ESS) and weakens an Exonic Splicing Enhancer (ESE)(Fig. 1c). To verify this hypothesis, we investigated the artificial c.2101 A > C change ($pATR^{2101C}$) that is predicted to affect the ESE only. Interestingly, this change gave rise to remarkably higher amounts (28 ± 7%) of correct transcripts (Fig. 1d) as compared to the SS-causing mutation. To further corroborate our findings, we created an antisense oligoribonucleotide (AON^{ATR}) masking the c.2101 A-including region. As shown in Fig. 1d, co-transfection of the AON^{ATR} improved exon 9 inclusion of both variants, particularly in the SS context (52 ± 10%), without showing a noticeable effect on the splicing pattern of the $pATR^{wt}$ minigene.

Taken together these findings demonstrate an interplay between ESS and ESE in governing the exon 9 definition, with a major role of the ESS that, once strengthened by the disease causing c.2101 A > G change, promotes exon skipping. It is worth noting that the mechanism involving an ESS in a weak exon does not appear to be uncommon, and has been described for disease-causing mutations in other genes [30–35].

Interestingly, the effect of the ESS vanished in the presence of strong 5'ss and 3'ss, thus confirming the crucial contribution of the splice site junction in driving exon 9 definition by the spliceosome. These

observations further highlight the fine balance between positive and negative regulatory elements governing the complex exon definition [36] that makes RNA processing very susceptible to derangements, as indicated by the growing number of disease-causing mutations through aberrant splicing [27–29]. On the other hand, our data with the AON^{ATR} provide an additional example of the potential of correction approaches based on antisense molecules, which have been successfully exploited by us and others in different disease models [25,37] and also in humans [38].

3.2. Strengthening recognition of the exon 9 5'ss by an engineered U1snRNA rescues ATR expression in cellular models

In the earliest splicing step, the 5'ss is recognized by the spliceosomal U1 small nuclear ribonucleoprotein (U1snRNP) by complementarity with the 5′ tail of its RNA component, the U1snRNA [26]. The low score of the authentic 5'ss of exon 9 is expected to weaken the U1snRNA binding, and accordingly its amelioration by mutagenesis restores exon inclusion. This observation prompted us to act *in trans* by exploiting a U1snRNA variant (U1^{ATR}) having the 5′ tail perfectly matching by complementarity the authentic exon 9 5'ss (Fig. 2a). This approach has been exploited by us and others in several human disease models, both *in vitro* and *in vivo*, to rescue splicing in the presence of mutations at the 5'ss, 3'ss or within exons [13–25].

Co-transfection of HEK293 cells expressing the *ATR* minigenes with the pU1^{ATR} remarkably rescued splicing in the wild-type (from 47 ± 4% to 96 ± 2% of correct transcripts) and, most importantly, in the presence of the SS-causing mutation (from 6 ± 1 to 62 ± 3%)(Fig. 2b). On the other hand, co-transfection of the pU1wt, mimicking overexpression of the endogenous one, had negligible effects in both conditions, thus indicating the functional role of the modified U1snRNA 5′ tail.

Altogether, these findings indicate the ability of the modified U1snRNA to efficiently rescue the SS mutation.

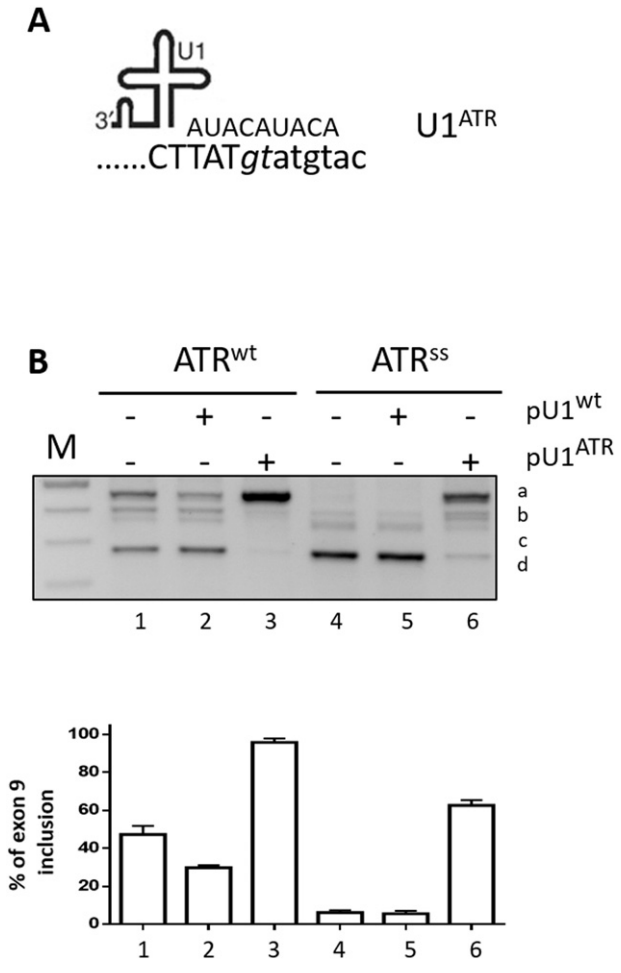

Fig. 2. Rescue of ATR splicing patterns by the modified U1^{ATR}. A) Sequences of the authentic *ATR* exon 9 5'ss and of the U1^{ATR} 5′ tail, with full complementarity. B) Evaluation of ATR alternative splicing patterns in HEK293 cells transfected with pATR minigenes alone (−), or in combination (+) with plasmids expressing the pU1 constructs. The normal and aberrant transcripts are indicated by letters (a-d) as in Fig. 1. Amplified products were separated on 2% agarose gel. M, 100 bp molecular weight marker. Histograms indicate the relative percentage of correctly spliced transcripts upon densitometric analysis of bands, which is expressed as mean ± standard deviation (SD) from at least three independent experiments.

3.3. The lentiviral-mediated U1^{ATR} delivery rescues ATR expression in humanized MEF^{SS} cells

To further assess its correction efficacy, the U1^{ATR} was challenged in embryonic fibroblasts from mice harboring the mutated human *ATR* cassette (MEF ATR$^{S/S}$), which recapitulates the ATR splicing profile observed in SS patients and the SS phenotype [7]. To overcome the very low transfection efficiency of these cells, which precluded us the direct exploitation of the AON^{ATR}, we packaged the U1^{ATR} cassette into a lentiviral vector (LV-U1^{ATR}, Fig. 3a, left). As shown in Fig. 3a (right panel), the infection resulted in the expression of the U1^{ATR} and, most importantly, in a partial rescue of ATR splicing, with correctly spliced transcripts raising from 19 ± 1 to 54 ± 3% (Fig. 3b). On the other hand, transduction with LV-U1^{ATR} did not change the splicing profile of mouse ATR in MEF cells from normal mice, thus indicating the U1 specificity toward the human 5'ss (Fig. 3b).

The U1^{ATR}-mediated rescue of ATR expression was also assessed at the intracellular protein level by Western Blotting. Noticeably, the ATR protein was observed in MEF ATR$^{S/S}$ cells only upon transduction with the LV-U1^{ATR}, and its levels raised to approximately 6% of the mouse ATR in control cells (Fig. 3c).

4. Conclusions

Taken together these findings elucidate the molecular mechanism underlying the defective ATR expression in the presence of the synonymous c.2101 A > G change, which causes Seckel Syndrome type 1. This led us to design two alternative approaches to modulate ATR splicing by AON or modified U1snRNA, both resulting in the remarkable rescue of the correct ATR transcripts, which could be exploited for other similar disease-causing mechanisms involving an ESS in a poorly defined exon [30–35].

It is worth noting that the AONs and the engineered U1snRNA, successfully explored for therapeutic purposes for other neurologic disorders [24], possess different features in an *in vivo* application perspective. AONs are easy to produce and deliver but have limited half-life and cellular specificity and permeability [39]. On the other hand, the U1snRNA have to be delivered by viral vectors such as adeno-associated virus [22,24], which can mediate cell target and guarantee long-lasting expression but can trigger an immune response. We are conscious that several issues have to be addressed (i.e. target tissues, time of treatment, clinical phenotype reversibility, etc) to evaluate the feasibility of these approaches for Seckel Syndrome therapy. Notwithstanding, the results obtained in this pioneer study lay the foundation for further studies in the available SS humanized mouse, a particularly complex model to develop RNA-based strategy for severe neuro-developmental disorders.

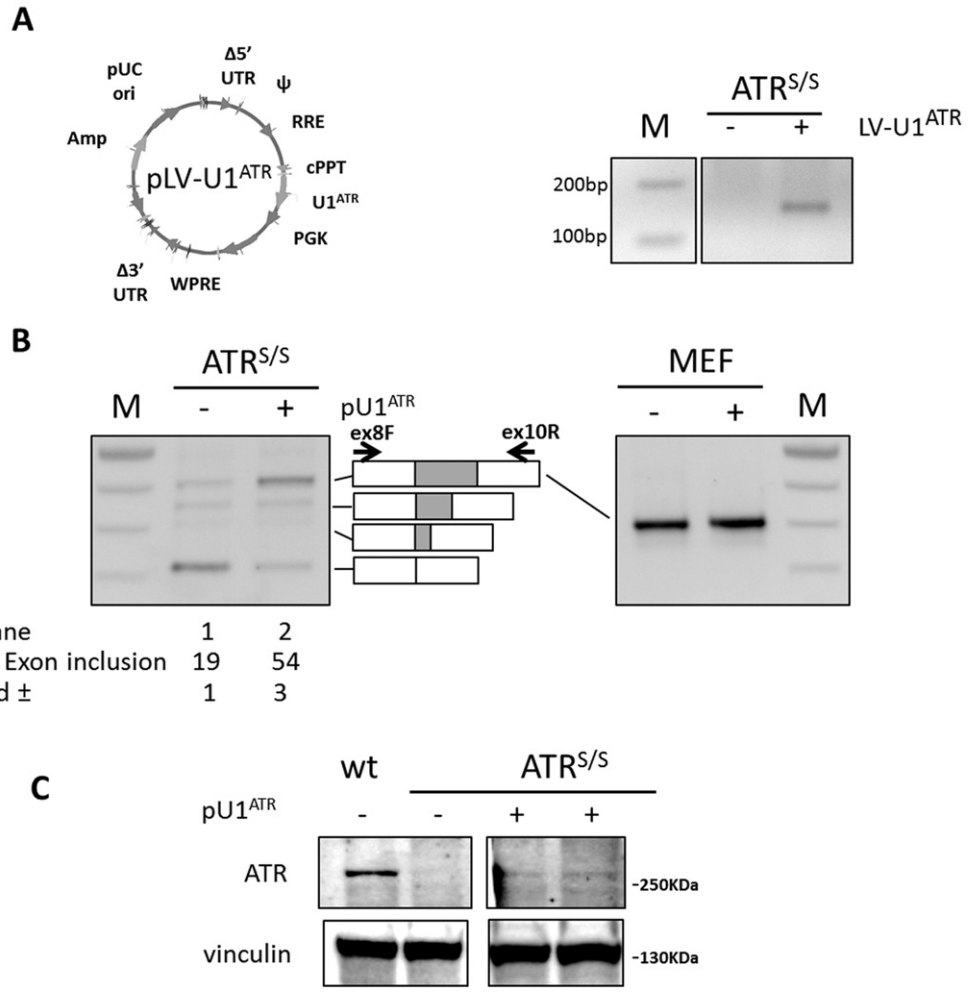

Fig. 3. Rescue of ATR expression in MEF$^{S/S}$ cells transduced with the LV-U1^{ATR}. A) Schematic representation of plasmid used to create the lentivirus vector and harboring the U1^{ATR} coding cassette (left panel), and RT-PCR based detection of the U1^{ATR} expression in MEF ATR$^{S/S}$ cells alone (−) or transduced (+) with the LV-U1^{ATR} (right panel). B) Evaluation of ATR alternative splicing patterns in MEF ATR$^{S/S}$ or MEF cells before (−) or after transduction (+) with LV-U1^{ATR}. The schematic representation of the normal and aberrant transcripts, and of primers used for the RT-PCR (arrows), is reported in the central panel. Amplified products were separated on 2% agarose gel. M, 100 bp molecular weight marker. The relative percentage of correctly spliced transcripts upon densitometric analysis of bands, expressed below as mean ± standard deviation (SD), is reported below. C) Evaluation of ATR expression by Western blotting analysis in MEF and MEF ATR$^{S/S}$ cells before (−) or after transduction (+) with LV-U1^{ATR}. Vinculin expression is used as loading control.

Conflicts of interest

D.S, D.B, S.R and S.C have no competing interests to declare. M.P. and F.B. are founders of the start-up company Raresplice.

Contributions

D.S. created the ATR minigenes, characterized the mechanism and performed the experiments with the AON^{ATR}; D.B. created the modified U1snRNA and assessed the rescue; S.R. created the LV vector, transduced MEF cells and evaluated ATR protein expression; M.B. designed the AON and revised the manuscript; D.P. synthesized the AON^{ATR}; F.B., S.C. and M.P. conceived the study, designed the experiments, analyzed and interpreted data and wrote the manuscript.

Transparency document

The Transparency document associated with this article can be found, in online version.

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