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Suppression of Nonsense Mutations in Rett Syndrome by Aminoglycoside Antibiotics

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ABSTRACT: Rett Syndrome (RTT) is caused in more than 60% of cases by nonsense mutations in the *MECP2* gene. So far, no curative therapy for RTT has become available. In other genetic disorders, it has been shown that aminoglycosides can cause a read-through of nonsense mutations with an efficiency of up to 20%. The aim of this study was to evaluate if this therapeutic concept is applicable to RTT. HeLa cells were transfected with eukaryotic expression vectors carrying mutant alleles of frequently occurring *MECP2* nonsense mutations that were N-terminally fused to a FLAG tag. Transfected cells were incubated 24 h in the presence of gentamicin. The expression of full-length protein was analyzed by Western blotting and immunofluorescent cell staining. In the presence of gentamicin a read-through varying between 10 and 21.8% was found, depending on the nucleotide sequence context of the nonsense mutations. The full-length protein was located correctly in the nucleus. We have shown that aminoglycoside-mediated read-through of nonsense mutations in the *MECP2* gene can be achieved *in vitro* with efficiency comparable with that seen in other disorders. **(*Pediatr Res* 65: 520–523, 2009)**

Rett syndrome (RTT) is an X-linked neurodevelopmental disorder that almost exclusively affects women (1). After normal development, during the first 6 to 18 mo developmental stagnation and then regression occurs. During the phase of regression, purposeful hand use and language are lost while gross motor functions are relatively preserved (2). After the phase of regression the clinical picture remains stable for many years. Although we have learned in recent years that RTT is caused by mutations in the *MECP2* gene, which codes for the methyl-CpG binding protein 2 (MeCP2), the unique developmental pattern remains to be explained (3). However, it is precisely this developmental pattern that gives hope that Rett syndrome is a treatable disorder. The fact that the affected girls have a period of normal development and after a phase of regression do not deteriorate any further distinguishes RTT from neurodegenerative disorders. Neuroanatomical studies also support the clinical finding that there is no neurodegeneration in RTT, aside from subtle changes in synaptic connectivity there are no brain malformations that would be clearly untreatable (4). Finally, the strongest argument that RTT might be a treatable disorder stems from the work of Guy *et al.* (5) who showed that in a mouse model of RTT the symptoms can be reversed by restoration of MeCP2 expression. Nevertheless, treating RTT is not simple for several reasons. First, MeCP2 seems to play a role in the control of expression of many genes and to date it is still not possible to specifically treat the downstream effects of the loss of MeCP2. Second, the description of men with severe mental retardation and spasticity caused by overexpression of MeCP2 due to duplications of the *MECP2* gene shows that the expression of MeCP2 is tightly controlled (6). The finding that overexpression is as deleterious as a loss of expression is supported by a mouse model where it causes a progressive neurologic disorder with seizures (7). These findings complicate the treatment of RTT as it is an X-chromosomal disorder where about half of the cells express a normal *MECP2* gene allele. Using a therapy that affects all cells, like protein replacement, would mean overdosing half of them.

Here, we describe studies of the aminoglycoside-mediated suppression of premature stop codons. This therapeutic approach is attractive for Rett syndrome, because it circumvents the problems mentioned above. The read-through only occurs in the cells where the mutated X-chromosome is expressed while the cells expressing the normal allele remain unaffected. It is also independent of the exact knowledge of the downstream effects of MeCP2 binding to the DNA and finally it would be applicable to a large percentage of patients because more than 60% of mutations found in RTT patients are nonsense mutations (8).

MATERIALS AND METHODS

Construction of eukaryotic expression vectors. Expression vectors encoding wild-type human MeCP2 and MeCP2 mutants bearing nonsense mutations identified in patients were constructed as follows. Full-length MeCP2 cDNA was amplified *via* PCR with cDNA generated from human fibroblasts and oligonucleotides containing restriction enzyme sites (MeCP2-Forw., 5′-ATAGAATTCAATGGTAGCTGGGATGTTAGGG-3′; MeCP2-Rev1., 5′-ATAGGATCCTCAGCTAACTCTCTCGGTCACGG-3′; MeCP2-Rev2., 5′-ATAGGATCCGCTAACTCTCTCGGTCACGG-3′). MeCP2 mutant cDNAs were generated by site-directed mutagenesis using PCR with mismatched primers (hMeCP2-502-C>T, 5′-GTATGATGTGTATT**T**GATCAATCCCCAGG-3′; hMeCP2-763-C>T, 5′-GCCCCGGCAGGAAG**T**GAAAAGCTGAGGCC-3′; hMeCP2-808-C>T, 5′-CCAAGAAACGGGGC**T**GAAAGCCGGGGAGTG-3′; hMeCP2-880-C>T, 5′-GGAGTCTTCTATC**T**GATCTGTGCAGGAG-3′) and the full-length MeCP2 cDNA. Synthesized wild type and mutant cDNAs were cloned into the eukaryotic expression vector p3×FLAG-CMV 7.1 and pN3-FLAG, respectively. Expression vector pN3-FLAG was generated by deleting EGFP from pEGFP-N3 where EGFP is enhanced green fluorescent protein (ConTech Laboratories, Palo Alto, CA) and inserting an oligonucleotide linker (N3-FLAG-

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Abbreviations: MeCP2, methyl-CpG binding protein 2; **RTT,** Rett syndrome

forw, 5′-GATCCGATTATAAAGATCATGACA TCGATTACAAGGATGACGATGACAAGTAGGC-3′; N3-FLAG-rev, 5′-GGCCGCCTACTTGTCATCGTCATCCTTGTAATCGATGTCATGATCTTTATAA TCG-3′). All constructs were verified by sequencing.

Cell culture. HeLa cells were maintained as monolayer culture growing in Dulbecco's modified Eagle's medium (DMEM/low glucose, Cambrex Bio Science, Verviers, Belgium) supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany) and 0.1% L-glutamine (PAA, Pasching, Austria). Cells were incubated at 37°C in an atmosphere of 5% CO_2.

Transfection and drug treatment. HeLa cells were transfected with the eukaryotic expression vectors using Effectene reagent (QIAGEN, Hilden, Germany) following manufacturer's protocols. A total of 3×10^5 HeLa cells were plated onto six-well culture plates and grown for 24 h. Transfection was performed with 400 ng of plasmid DNA for 8 h in fresh media and media containing either 1 mg/mL gentamicin (SIGMA, Taufkirchen, Germany) or 100 μg/mL geniticine (Roth, Karlsruhe, Germany). After transfection, cells were washed once with phosphate-buffered saline (PBS) (PAA, Pasching, Austria), and incubated in fresh media and media containing 1 mg/mL gentamicin for 24 h. Transfection efficiency was determined by analyzing total cell number (DAPI (4′,6-Diamidino-2-phenylindole) staining) and number of transfected cells (FLAG staining). An effect of drug treatment on endogenous MeCP2 expression was excluded by gentamicin and geniticine (G418) treatment of untransfected cells (Fig. 1*A*).

Preparation of cell lysates. Gentamicin-treated and -untreated transfected HeLa cells plated in six-well culture plates were rinsed twice with PBS and lysed on ice in 150 μL RIPA (radio immunoprecipitation assay buffer) buffer [150 mM NaCl; 50 mM Tris/HCl (pH 8.0); 5 mM EDTA (pH 8.0); 0.8% sodium deoxycholate; 1% Nonidet P-40; 0.1% SDS] containing 0.1% (vol/vol) of a stock solution of the proteinase inhibitor cocktail Complete (Roche Diagnostics, Heidelberg, Germany) per well for 15 min. Lysates were centrifuged at 4°C (18,000*g* for 15 min) to remove insoluble matter. The concentration of protein in the supernatant fractions was determined using the BC assay (Uptima, Montlucon cedex, France), following manufacturer's protocols.

Western blotting analysis. Cell lysates were prepared for electrophoresis by addition of sample buffer (ROTI-Load1, 4× concentrated; Roth, Karlsruhe, Germany) and heating to 95°C for 5 min. A total of 30 μg of each protein sample were separated on a 10% SDS-polyacrylamid gel, and transferred onto nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Nonspecific binding sites were blocked by incubation with blocking solution [5% nonfat milk powder in PBST (phosphate buffered saline Tween-20) (1× PBS/0.1% Tween 20)] for 1 h at room temperature (RT), after an overnight incubation with specific primary antibodies diluted in blocking solution at 4°C (monoclonal anti-FLAG M2, SIGMA, Taufkirchen, Germany; anti-MeCP2 detecting the N-term; Acris Antibodies, Hiddenhausen, Germany, anti-MeCP2 detecting the C-term, Etienne Joly, Toulouse, France). As secondary antibodies, horseradish-peroxidase-conjugated donkey anti-mouse IgG and goat anti-rabbit IgG were used (Jackson Immuno-Research Laboratories, West Grove, PA) diluted in PBST. All antibodies were used at concentrations recommended by the manufacturer. Immunoreactive bands were visualized using enhanced chemiluminescence (Lumi-Light Western Blotting Substrate, Roche Diagnostics, Heidelberg, Germany). The efficiency of the read-through was estimated in comparing the expression of total MeCP2 with the expression of full-length protein using densitometry (Camera: Fuji LAS-1000plus; Programm: Aida Version 3.10).

Immunofluorescent cell staining. HeLa cells were grown and transfected on 18-mm cover slips. Twenty-four hours after transfection, cells were washed once with PBS and fixed in 4% paraformaldehyde containing 4% sucrose for 20 min at RT. After washing three additional times with PBS, the cells were permeabilized with 0.25% Triton X-100/PBS for 5 min at RT, washed again three times with PBS and blocked with 10% BSA/PBS at 37°C for 30 min. After an overnight incubation with a monoclonal anti-FLAG M2 antibody (SIGMA, Taufkrichen, Germany; 1:1000 dilution in 3% BSA/PBS), cells were washed three times with PBS and incubated with an Alexa Fluor 488 conjugated anti-mouse IgG antibody (Molecular Probes Inc., Eugene, OR; 1:500 dilution in 3% BSA/PBS) at 37°C for 1 h. The prepared cover slips were mounted onto slides with ProLong Gold antifade reagent with DAPI (Molecular Probes Inc., Eugene, OR). The stained cells were analyzed using fluorescent microscopy (Zeiss, Göttingen, Germany).

The study has been approved by the institutional review board of the Georg August University, Göttingen, Germany.

RESULTS

To determine whether gentamicin can induce the functional suppression of disease-causing nonsense mutations in human cells, we assayed the synthesis of full-length MeCP2 from cDNAs containing four naturally occurring premature stop mutations that cause Rett syndrome. In all cases, the mutations introduce an in-frame ochre (UGA) stop codon in place of arginine residue 168, 255, 270, and 294 of MeCP2, which result in UGAG, UGAA, UGAA, and UGAU tetranucleotide termination signals, respectively.

HeLa cells were transfected with eukaryotic expression vectors carrying both the wild-type *MECP2* cDNA and mutated *MECP2* cDNAs N-terminally fused to a FLAG-tag. After transfection, the full-length FLAG-MeCP2 fusion protein and the truncated FLAG-tagged isoforms were expressed. The full-length MeCP2 was absent in cells transfected with *MECP2* cDNAs containing nonsense mutations showing that read-through of these premature stop mutations does not occur under normal conditions (Fig. 1).

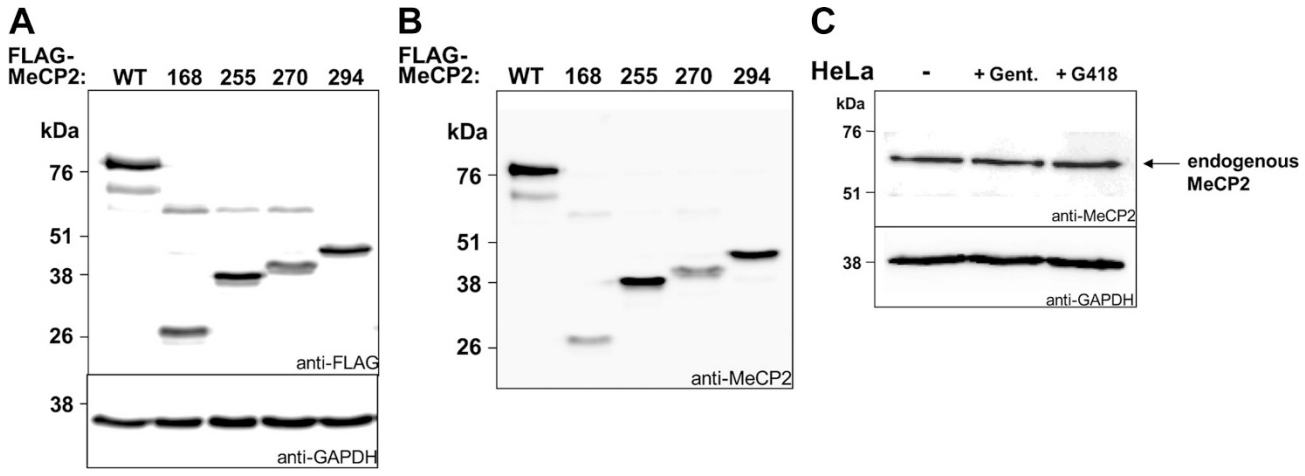

Figure 1. Expression of FLAG-MeCP2 fusion proteins in transfected HeLa cells. Immunblot analysis of protein lysates prepared from transfected HeLa cells. FLAG-MeCP2 fusion proteins expressing WT and mutated MeCP2-isoforms were detected using a monoclonal anti-FLAG antibody (*A*) and a specific anti-MeCP2 antibody detecting the N-terminal part of MeCP2 protein (*B*). Analysis of endogenous MeCP2 expression in nontransfected HeLa cells nontreated or treated with either gentamicin or geniticine using a specific anti-MeCP2 antibody detecting the C-terminal part of MeCP2 (*C*). As a loading control, expression of the housekeeping gene GABDH was detected in all samples.

We next assayed whether the suppression of these nonsense mutations could be stimulated by pharmacologic treatment. We incubated HeLa cells transfected with the *MECP2*-R168X, -R255X, -R270X, and -R294X constructs in the presence of 1 mg/mL of gentamicin for 24 h. Transfection efficiency was 25–30% in nontreated cell and 22–28% in the gentamicin-treated cells. We were able to detect full-length MeCP2 expression in all four cell lines containing the stop mutations R168X, R255X, R270, and R294X using an anti-FLAG and an anti-MeCP2 N-terminal antibody (Fig. 2). The efficiency was lowest for the R168X mutation (10%) and highest for the R294X mutation (21.8%), whereas R255X (13.5%) and R270X (11.8%) were intermediate. Repeating the experiments using geniticine, another aminoglycoside that has been found to cause read-through of nonsense mutations, instead of gentamycin, we found similar read-through efficiency (Fig. 2*D*). To determine whether lower gentamicin concentrations could lead to suppression of the premature stop codons, HeLa cells were transfected with the aforementioned different constructs and cultured in the presence of various gentamicin doses ranging from 0 to 800 μg/mL for 24 h. Using an anti-FLAG antibody, full-length MeCP2 expression was already detected at the lowest concentration of 50 μg/mL. Higher read-through efficiency could be achieved with increasing gentamicin concentrations. The expression level seems to depend on the kind of mutation (Fig. 3).

To analyze the location of the MeCP2 proteins that resulted from the read-through in the cell, we performed immunofluorescent cell staining using a monoclonal anti-FLAG M2 antibody. Therefore, HeLa cells were transfected with eukaryotic expression vectors carrying both the wild-type *MECP2* cDNA and mutated *MECP2* cDNAs C-terminally fused to a FLAG-tag. After transfection, the full-length FLAG-MeCP2 fusion protein and the truncated FLAG-tagged isoforms were expressed. It showed that after gentamicin treatment the read-through (full-length) protein is located in the nucleus like the wild-type FLAGG-tagged MeCP2 protein (Fig. 4).

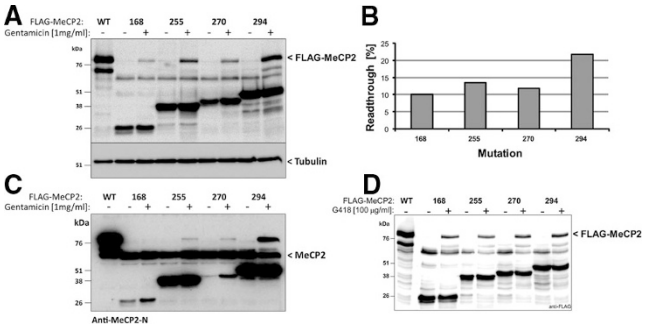

Figure 2. Gentamicin suppresses premature nonsense mutations. Western blot analysis of protein samples prepared from gentamicin-treated (1 mg/mL for 24 h) (*A*) and geniticine-treated (100 μg/mL for 24 h) (*D*) and untreated transfected HeLa cells, using a monoclonal anti-FLAG antibody. Thirty micrograms of protein extract were loaded into each lane. As a loading control, tubulin was detected in all samples. The effect of 24 h gentamicin treatment was quantified by densitometric-developed Western blot analysis ($n = 3$) (*B*). MeCP2 protein expression was verified by using an MeCP2-specific antibody detecting the N-terminus of MeCP2 (*C*).

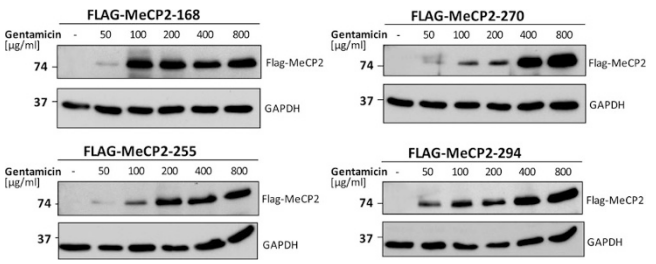

Figure 3. Read-through efficiency depends on gentamicin concentration and type of nonsense mutation. Western blot analysis of protein samples prepared from gentamicin-treated (0–800 μg/mL for 24 h) and untreated transfected HeLa cells, using a monoclonal anti-FLAG antibody. Thirty micrograms of protein extract were loaded into each lane. As a loading control, GABDH was detected in all samples.

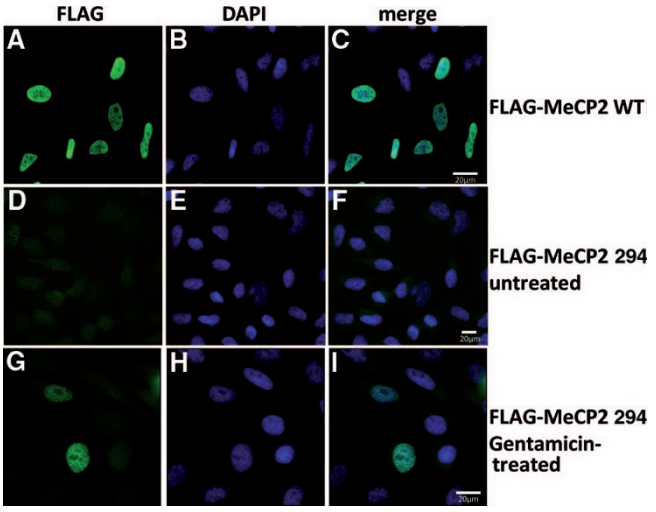

Figure 4. Full-length MeCP2-FLAG fusion protein is expressed in gentamicin-treated HeLa cells. (*A–C*) Immunohistochemistry of HeLa cells transfected with MeCP2-WT-FLAG. (*D–F*) HeLa cells transfected with MeCP2-294-FLAG, untreated. (*G–I*) HeLa cells transfected with MeCP2-294-FLAG, treated with gentamicin (1 mg/mL for 24 h). Localization of FLAG fusion proteins (*left panel*) was visualized by using a monoclonal anti-FLAG antibody and immunofluorescence microscopy and the nuclei were counterstained with DAPI (*middle panel*). *Left panel*, MeCP2-WT-FLAG expression as well as treated MeCP2-294-FLAG overlaps with DAPI staining, indicating a nuclear localization.

DISCUSSION

In the presence of high concentrations of aminoglycosides that bind to the 16S rRNA, the ribosomal proofreading is impaired leading to a read-through of nonsense mutations (9–11). Instead of the nonfunctional shortened protein, a full-length protein is produced that incorporates a random amino acid at the position of the stop codon. In several different genetic disorders, this therapeutic strategy has been tested, but so far, it has not been incorporated into routine therapeutic practice (9,10,12,13). There are several drawbacks associated with this therapeutic strategy. First, the read-through of nonsense mutations is not complete but with an efficiency varying between 1 and 20% depending on context of the stop codon, and second, the high concentrations of aminoglycosides needed to reach notable efficiency will cause side effects if administered permanently (14). Nevertheless, RTT might be a good candidate for this therapy as MeCP2 is

involved in the long-term silencing of genes and might therefore have a relatively long-life span. Furthermore, the therapy would be applicable to many patients because 60% of all cases of RTT are caused by nonsense mutations (15).

Because it has been shown that the read-through is most effective in the UGA stop codon, we chose four stop mutations R168X, R255X, R270X, and R294X that occur frequently in RTT (16). While mutation R168X truncates the protein between the methyl-binding domain and the transcriptional expression domain, the remaining mutations occur at different positions in the more C-terminal located transcriptional repressor domain. In our experiments, we were able to induce read-through with efficiency between 10 and 22%. Our results confirm the finding of Howard *et al.* (16) who demonstrated that the efficiency of the read-through in eukaryotic cells is dependent on the context surrounding the stop codon. The read-through was more effective when the stop codon is followed by U and less effective when followed by G while A is intermediate. We also demonstrated that the read-through is dosage dependent. Using immunofluorescent cell staining we were able to show that the full-length protein is transported into the nucleus. In conclusion, our results show that in cell culture an effective read-through of nonsense mutations in *MECP2* by gentamicin is possible and that the full-length protein is transported into the nucleus. These data only prove the concept. So far, we do not know if the full-length protein is biologically active and if the efficiency of the read-through is high enough to prevent pathologic effects. These questions will have to be addressed in an animal model. These animal studies will also show if the blood-brain barrier allows sufficient concentrations of gentamicin to cross into the brain for effective read-through. However, if gentamicin proves to be ineffective or toxic, other substances that follow the same therapeutic pathway should be tested. Only recently, PTC124, a drug that is capable of suppressing premature termination at a higher level than the aminogylcosides and without obvious side effects, has been described and might be a good candidate for therapy in RTT (17).

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