

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/5774845>

# Drug-induced readthrough of premature stop codons leads to the stabilization of laminin $\alpha 2$ chain mRNA in CMD myotubes

ARTICLE *in* THE JOURNAL OF GENE MEDICINE · FEBRUARY 2008

Impact Factor: 2.47 · DOI: 10.1002/jgm.1140 · Source: PubMed

CITATIONS

44

READS

131

13 AUTHORS, INCLUDING:



[Masataka Shiozuka](#)

Kyushu University

24 PUBLICATIONS 198 CITATIONS

[SEE PROFILE](#)



[Gillian Butler-Browne](#)

Pierre and Marie Curie University - Paris 6

301 PUBLICATIONS 10,076 CITATIONS

[SEE PROFILE](#)



[Ryoichi Matsuda](#)

The University of Tokyo

84 PUBLICATIONS 1,643 CITATIONS

[SEE PROFILE](#)



[Pascale Guicheney](#)

Pierre and Marie Curie University - Paris 6

339 PUBLICATIONS 12,172 CITATIONS

[SEE PROFILE](#)

# Drug-induced readthrough of premature stop codons leads to the stabilization of laminin $\alpha 2$ chain mRNA in CMD myotubes

Valérie Allamand<sup>1,2\*</sup>,<sup>†</sup> Laure Bidou<sup>3,4†</sup> Masayuki Arakawa<sup>5</sup> Célia Floquet<sup>3,4</sup> Masataka Shiozuka<sup>5</sup> Marion Paturneau-Jouas<sup>1,2</sup> Corine Gartiaux<sup>1,2</sup> Gillian S. Butler-Browne<sup>6</sup> Vincent Mouly<sup>6</sup> Jean-Pierre Rousset<sup>3,4</sup> Ryoichi Matsuda<sup>5</sup> Daishiro Ikeda<sup>7</sup> Pascale Guicheney<sup>1,2</sup>

<sup>1</sup>Inserm, U582, Paris, France

<sup>2</sup>Université Pierre et Marie

Curie-Paris 6, UMR-S582, Institut de Myologie, IFR14, Paris, France

<sup>3</sup>IGM, Université Paris-Sud, UMR 8621,

Orsay, F-91405, France

<sup>4</sup>CNRS, Orsay, F-91405, France

<sup>5</sup>Department of Life Sciences, The University of

Tokyo at Komaba., 3-8-1 Komaba,

Meguro-ku, Tokyo 153-8902, Japan

<sup>6</sup>Inserm UMR-S787, Institut de

Myologie, Faculté de Médecine

Pitié-Salpêtrière, Paris, F-75013,

France

<sup>7</sup>Microbial Chemistry Research

Center, Numazu Bio-Medical Research

Institute, 18–24 Miyamoto, Numazu

City, Shizuoka 410-0301, Japan

\*Correspondence to: Valérie Allamand, Inserm U582, Institut de Myologie, Groupe Hospitalier Pitié-Salpêtrière, 47 Bd de l'Hôpital, F-75651 Paris Cedex 13, France.

E-mail:

v.allamand@institut-myologie.org

<sup>†</sup>These authors contributed equally to this work.

## Abstract

**Background** The most common form of congenital muscular dystrophy is caused by a deficiency in the  $\alpha 2$  chain of laminin-211, a protein of the extracellular matrix. A wide variety of mutations, including 20 to 30% of nonsense mutations, have been identified in the corresponding gene, *LAMA2*. A promising approach for the treatment of genetic disorders due to premature termination codons (PTCs) is the use of drugs to force stop codon readthrough.

**Methods** Here, we analyzed the effects of two compounds on a PTC in the *LAMA2* gene that targets the mRNA to nonsense-mediated RNA decay, *in vitro* using a dual reporter assay, as well as *ex vivo* in patient-derived myotubes.

**Results** We first showed that both gentamicin and negamycin promote significant readthrough of this PTC. We then demonstrated that the mutant mRNAs were strongly stabilized in patient-derived myotubes after administration of negamycin, but not gentamicin. Nevertheless, neither treatment allowed re-expression of the laminin  $\alpha 2$ -chain protein, pointing to problems that may have arisen at the translational or post-translational levels.

**Conclusions** Taken together, our results emphasize that achievement of a clinical benefit upon treatment with novel readthrough-inducing agents would require several favourable conditions including PTC nucleotide context, intrinsic and induced stability of mRNA and correct synthesis of a full-length active protein. Copyright © 2007 John Wiley & Sons, Ltd.

**Keywords** congenital muscular dystrophy; laminin  $\alpha 2$  chain; premature termination codon; antibiotic-mediated readthrough; nonsense-mediated mRNA decay

## Introduction

Congenital muscular dystrophy (CMD) is a clinically and genetically heterogeneous group of neuromuscular disorders with autosomal recessive inheritance. The 'classic' (or occidental) form of CMD is characterized by clinical manifestations mainly affecting skeletal muscle [1–4]. A specific deficiency of the  $\alpha 2$  chain of laminin-211 is responsible for about 30 to 40% of these cases (MDC1A, MIM#607855). Numerous mutations have now been identified in the *LAMA2* gene encoding the  $\alpha 2$  chain of laminin, leading to either complete or partial protein deficiency [5–9]. Notably, 20 to 30% of the mutations identified in the *LAMA2* gene are nonsense mutations leading to a premature termination codon (PTC).

Received: 6 July 2007

Revised: 17 September 2007

Accepted: 19 October 2007

Thus, up to 15% of 'classical' CMD patients would potentially benefit from a pharmacological strategy designed to promote translation of the endogenous *LAMA2* mRNA interrupted by a PTC. From early work in *Escherichia coli*, it has been demonstrated that aminoglycoside antibiotics such as gentamicin can specifically act on ribosomal RNA to suppress codon-anticodon recognition by aminoacyl tRNAs, thereby interfering with the translation termination process and suppressing stop codons [10–14]. Aminoglycoside-mediated translational readthrough of PTCs involved in several human genetic disorders, including Duchenne muscular dystrophy, has now been reported with variable degrees of success [15–26]. It has also become increasingly evident that only a small subset of stop codon mutations would benefit from gentamicin treatment, depending on their nucleotide context [21,27–30]. In addition, strong side effects of gentamicin, the most commonly used aminoglycoside antibiotic, have been well documented [31,32] and hamper its utilization as a potential therapeutic agent especially if long-term treatment is required. Furthermore, gentamicin-induced readthrough is dependent on its chemical composition and its reproducibility is debatable [33].

To address these problems, we tested two other compounds with antibiotic activities, negamycin and amikacin [34], known to provide effective suppression of nonsense mutations in cellular and animal models and to present a lower toxicity than gentamicin [20,34–38]. Unlike gentamicin and amikacin, that belong to the aminoglycoside family, negamycin is a dipeptide antibiotic, which also interacts with the ribosomal A site to mediate readthrough [38].

Due to the absence of an adequate animal model for MDC1A, we chose an *ex vivo* approach based on muscle explants obtained from a patient presenting a complete merosin deficiency due to a homozygous nonsense mutation (c.C4687A) in exon 31 of the *LAMA2* gene causing a PTC (C1546X) [39]. We treated myotubes derived from this patient with either gentamicin or negamycin. Since readthrough efficiency depends on the nature of the PTC and its surrounding sequences [21,28,40–43], in parallel we measured termination readthrough for this specific *LAMA2* mutation, in its nucleotidic context, in a sensitive and reproducible dual reporter assay [40,43]. In addition, the presence of PTCs often results in the rapid degradation of the mutant mRNA by the nonsense-mediated mRNA decay (NMD) pathway (for a review, see [44]). It has been suggested that gentamicin, by allowing some translational readthrough, may result in reduced levels of NMD [14,45].

In this study, using a dual reporter assay, we first demonstrated, *ex vivo* as well as *in vivo*, that negamycin is as effective as gentamicin in inducing PTC readthrough. Interestingly, negamycin-induced readthrough of the *LAMA2* PTC studied here was among the highest levels reached using this assay. Moreover, by quantitative reverse-transcription polymerase chain reaction (RT-PCR) we demonstrated that negamycin treatment, in contrast

to gentamicin, strongly stabilized the patient's laminin  $\alpha 2$ -chain mRNA levels. Indeed, mutant mRNA levels were significantly decreased in the untreated patient's cells, likely due to the NMD pathway. However, re-expression of the laminin  $\alpha 2$ -chain protein could not be detected in our experimental conditions.

In conclusion, our data demonstrate that for some mutations, stabilization of the mutant mRNA can be obtained through a sufficient level of readthrough, which, in some cases, could allow a synergistic effect on PTC suppression. Nonetheless, this may not be sufficient to allow re-expression of a functional full-length protein, suggesting that numerous steps need to be fulfilled to obtain a clinical benefit through PTC-induced suppression.

## Materials and methods

### Cell culture and transfection

NIH3T3 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-Gibco, Cergy, France) supplemented with 7% fetal calf serum (Gibco BRL), and incubated at 37°C in humidity saturated 6.5% CO<sub>2</sub>. Cells were electroporated with 18 µg of each pAC(lacZ-luc) plasmid construct, as described [21], and plated immediately in culture medium without antibiotic. After 20 h and 28 h the medium was replaced with fresh medium complemented or not with gentamicin (Invitrogen-Gibco, Cergy, France), negamycin (both used at 600 µg/ml) or amikacin (GIBCO BRL; at 2mg/ml). Two days after transfection, cells were harvested, spun for 5 min at 4°C and lysed by repeated pipetting in 150 µl of cold luciferase assay buffer. For each construct, at least five independent transfection experiments were performed.

### Reporter plasmid cloning and stop mutation targets

Complementary oligonucleotides corresponding to the sequence of each stop mutation embedded in its natural nucleotide context were annealed and cloned in a dual gene reporter system between the lacZ and luc coding sequences as previously described [21]. The nucleotidic context around the C1546X stop mutation sequence is: 5' GTC ACA GGA TTC TGA ACG TGC CGA CCT 3'. For the other stop targets oligonucleotides sequences were previously published [21].

### Enzyme assays

Luciferase and  $\beta$ -galactosidase activities were measured from the same crude extract as described [43]. Stop codon readthrough was calculated by dividing the ratio of luciferase to  $\beta$ -galactosidase activity obtained with a given test construct by the ratio obtained from the control

construct where the stop codon was replaced by a sense codon. Each value of readthrough efficiency corresponds to the mean of five to six independent experiments. Standard deviation errors did not exceed 20%.

## Cell culture and immunohistochemistry

Primary myoblasts obtained from a control foetus (12 weeks of gestation) and a merosin-deficient CMD foetus (15 weeks of gestation), presenting a homozygous nonsense mutation in exon 31 of the *LAMA2* gene, were grown in F10 medium + 20% SVF + penicillin/streptomycin (10 U/ml and 10 µg/ml, respectively; Invitrogen-Gibco). Muscle cells were obtained in accordance with the French legislation on ethical rules. Myoblasts were allowed to fuse for 7 days on 0.5% gelatin-coated coverslips in antibiotic-free or negamycin-containing (300 µg/ml; Institute of Microbiology, Tokyo, Japan) differentiation medium (DMEM 4.5 g glucose + 2% horse serum +  $10^{-6}$  M insulin +  $2.5 \times 10^{-6}$  M dexamethasone). Immunohistochemical analyses were performed on methanol-fixed cells (10 min at 4°C) as follows: non-specific sites were blocked for 30 min in 5% bovine serum albumin (BSA)/1X phosphate-buffered saline (PBS), and cells were then incubated overnight at 4°C with primary antibodies against myosin (MF20, a gift from Dr. Denis Furling, Inserm-UMR S 787, Institut de Myologie, Paris, France) and laminin  $\alpha 2$  chain (4H8-2, Alexis Biochemicals, Lausen, Switzerland) diluted into 1% BSA/1X PBS. Following three washes in 1% BSA/1X PBS, cells were incubated for 90 min in appropriate FITC or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). After three additional washes, cells were mounted with Mowiol containing DAPI (0.05 µg/µl final) to stain all of the nuclei and observed under a Zeiss microscope. Images were captured under identical conditions using the Metaview software (Ropper Scientific GmbH, Germany).

## RNA extraction and quantitative RT-PCR

Total RNAs were extracted using conventional TRIzol™ reagent (Life Technologies, Inc.) extraction protocol as recommended by the manufacturer. RNA quality control and quantification was performed using the Bioanalyzer apparatus (Agilent). First strand cDNA was synthesized from 1 µg of total RNA using oligo(dT)<sub>18</sub> and Superscript reverse transcriptase II (Invitrogen-Gibco, Cergy, France) as recommended by the manufacturer. Quantitative PCR was then carried out on equal amounts of cDNAs, using the LightCycler real-time PCR machine (Roche Diagnostics, Germany), and *LAMA2* mRNA levels were quantified relative to the mRNA of the troponin T1 (*TPNT1*) gene. In each experiment, results were expressed relative to control myotubes normalized to 100. The oligonucleotide pairs used for the amplifications are as

follows: *TPNT1* forward: 5' TGGAGCTGCAGACACTCATC 3' and reverse: 5' GCTTCTGTTCTGCCTTGACC 3'; *LAMA2* forward: 5' TGTGCTGCAGAATCAGAACC 3' and reverse: 5' ATTGATTTTGGTGGGGATCA 3'. Mean values of several quantitative RT-PCRs (n = 4) are presented + standard deviation to mean. Statistical significance was determined by a t-test (\*p < 0.001).

## Protein extraction and immunodetection

Total proteins were extracted in SDS buffer (80 mM Tris-HCl, pH6.8, 10% SDS, 0.12 M sucrose, 10 mM EDTA, 1 mM PMSF, 1 mM benzamidine). Concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with BSA as a standard. Proteins were subsequently resolved under reducing conditions by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and transferred to PVDF membranes. Detection of the laminin  $\alpha 2$  chain was performed by using an affinity purified polyclonal antibody (rabbit 180, a gift from Dr. Kevin P. Campbell, Molecular Physiology and Biophysics, Howard Hughes Medical Institute, Iowa City, USA). HRP-conjugated secondary antibody anti-rabbit IgG (DakoCytomation, Glostrup, Denmark) was used at a dilution of 1:2000. Immunoblots were developed using enhanced chemiluminescence (SuperSignal West Pico chemiluminescent substrate; Pierce, Rockford, IL). In order to control the amounts of proteins which had been loaded, the gel was stained with Coomassie blue following transfer.

## In vivo studies

All animal studies conformed to the guidelines on animal use procedures approved by Inserm. Mice were anesthetized by intraperitoneal injection of 10 mg/ml ketamine (Clorketam 1000; Vetoquinols SA, Lure, France) and 5 mg/ml xylazine (Rompun 2%; Bayer Pharma, Puteaux, France). Plasmid DNA (35 µg) was injected percutaneously into the tibialis anterior muscle. Two minutes after the injection, transcutaneous electric pulses were applied through external stainless steel plates (200 V/cm, 8 pulses of 20 ms each, 2 Hz) on a ECM 830 BTX electroporator (San Diego, USA).

Animals were subsequently treated with gentamicin (34 mg/kg; Invitrogen-Gibco) or PBS once daily for 3 days, by percutaneous injections. On the last day, mice were euthanized and the injected muscles were harvested.

## Results

### Negamycin promotes readthrough in a dual reporter assay in NIH3T3 cells

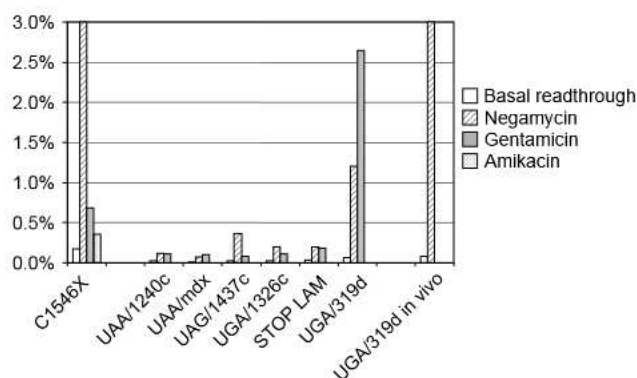
The C1546X mutation in the *LAMA2* gene leads to a UGA premature termination codon (PTC) which has been



reported to be the most sensitive codon to aminoglycoside-induced readthrough [28,41,42]. Using a previously published dual reporter assay [40], we demonstrated that this PTC is responsive to amikacin and gentamicin (0.4% and 0.6%, respectively, which correspond to 2.5- and 3.8-fold increases in readthrough; Figure 1). Interestingly, readthrough was increased up to 3% (19-fold) following negamycin treatment which is among the highest levels we have found using this assay [21]. To examine more precisely the effect of negamycin on other stop targets, five other nonsense mutations leading to PTCs in either the human or murine dystrophin genes (UGA/319d and UAA/mdx, respectively) and the *LAMA2* gene (UGA/1326c, UAG/1437c, UAA/1240c), as well as the natural termination codon of the *LAMA2* gene (UGA/STOP LAM), were tested under the same conditions. These targets have previously been analyzed for gentamicin sensitivity [21]. For six of the seven stop targets presented in Figure 1, negamycin is equally or more effective than gentamicin in promoting readthrough. Nevertheless, in five of these targets the efficiency remained modest (0.4%; 2.5-fold). Only one stop mutation (UGA/319d) was more sensitive to gentamicin (2.6%, 44-fold) than to negamycin (1.2%, 20-fold). Thus, among six mutations only two can be highly bypassed by antibiotic treatment: C1546X by negamycin and UGA/319d by gentamicin; the latter also being moderately bypassed by negamycin.

### Negamycin allows readthrough *in vivo*

Since the availability and metabolism of negamycin might be modulated *in vivo*, the results in cultured cells might not reflect exactly the situation in muscles.



**Figure 1.** Induction of readthrough in NIH3T3 cells. Sequences corresponding to the C1546X stop mutation as well as other stop targets corresponding to DMC (c) or DMD (d) stop mutations or to the natural termination codon of the *LAMA2* gene (UGA/STOP LAM) were cloned in a dual gene reporter system between lacZ and luc coding sequences. NIH3T3 cells were treated with gentamicin, negamycin or amikacin. Termination readthrough is expressed as the luciferase/ $\beta$ -galactosidase ratio of the test construct normalized to an in-frame control where stop codons have been replaced by a sense codon. Each value of readthrough efficiency corresponds to the mean of four to six independent experiments. Standard deviation errors did not exceed 20%

As previously shown for gentamicin [21], we assessed the effect of negamycin *in vivo*, in C57BL/6 mice. The vector carrying the UGA/319d mutation was injected into skeletal muscle with subsequent electrotransfer and thereafter mice were treated intramuscularly once daily with negamycin (34 mg/kg) or PBS for the following 3 days. Results showed that both basal and negamycin-induced readthrough efficiency were similar in NIH3T3 cells and *in vivo*, increasing from 0.08% in the absence to 3% (38-fold) in the presence of the drug (Figure 1).

### Stabilization of *LAMA2* mRNA in negamycin-treated myotubes

The mutation in exon 31 of the *LAMA2* gene leads to the replacement of a cysteine residue by a premature UGA stop (C1546X) that is placed more than 50 nucleotides upstream of the 3' most exon-exon junction and is therefore in a favourable condition for the degradation of the mutant mRNA by the NMD pathway [44]. Indeed, quantitative RT-PCR analysis demonstrated a significant decrease in the level of *LAMA2* transcripts which were reduced to 6.25% of control values (Figure 2). We hypothesized that, by promoting translational readthrough of the mutant mRNA, gentamicin and negamycin would prevent its rapid degradation by the NMD pathway. Treatment of the patient's myotubes by two different batches of gentamicin (GIBCO BRL or American Pharmaceutical Partners, Inc.) did not trigger a significant increase in the level of transcripts. However, negamycin treatment strongly sustained the stabilization of the *LAMA2* transcripts up to 77% of control levels (Figure 2). These results are in agreement with those obtained in *S. cerevisiae* where previous studies indicated an inverse relationship between readthrough level and NMD-dependent decrease in mRNA abundance [20,46]. Moreover, the mRNA quantification data can be correlated to those obtained in our cell culture expression system in which negamycin was more efficient than gentamicin in promoting translational readthrough of the *LAMA2* nonsense mutation (Figure 1).

### Neither gentamicin nor negamycin enabled re-expression of laminin $\alpha 2$ chain in MDC1A myotubes

In parallel to the mRNA studies, we investigated whether gentamicin or negamycin treatment induced re-expression of the laminin  $\alpha 2$  chain in the patients' myotubes at the protein level. Control and MDC1A myoblasts were allowed to differentiate *ex vivo* for 7 days and subsequently myotubes were treated with gentamicin (GIBCO BRL or American Pharmaceutical Partners, Inc.) or negamycin for 7 or 15 days. Expression of myosin and the laminin  $\alpha 2$  chain was then assessed on fixed myotubes by immunohistochemistry (Figure 3A). First, we observed that MDC1A myoblasts were indeed able to efficiently differentiate into myotubes as indicated by

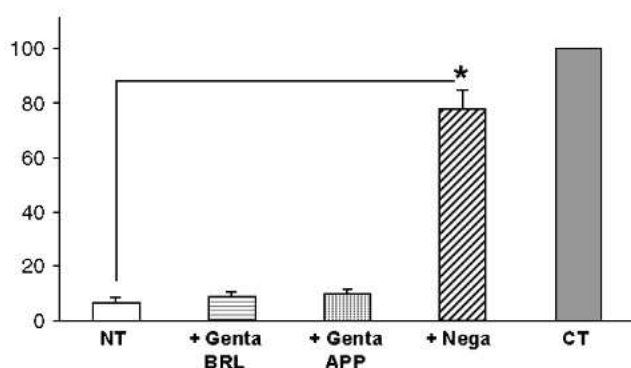


Figure 2. LAMA2 mRNA levels in cultured MDC1A myotubes treated with gentamicin and negamycin. Quantitative PCR were carried out on equal amounts of cDNAs, using the LightCycler real-time PCR machine (Roche Diagnostics, Germany). LAMA2 mRNA levels were quantified relative to the mRNA of the troponin T1 (TPNT1) gene. In each experiment, results were expressed relative to control myotubes normalized to 100. Mean values are presented  $\pm$  standard deviation to mean. Statistical significance was determined by a t-test ( $p < 0.001$ )

the strong expression of myosin (panel d) which was comparable to that observed in the control cells (panel a). However, immunohistochemical analyses did not allow detection of the laminin  $\alpha 2$  chain following either a 7- or 15-day-long treatment with doses as high as 300  $\mu\text{g}/\text{ml}$  of gentamicin or negamycin (panels h, i and data not shown). Biochemical analyses confirmed these results for all three compounds tested (Figure 3B), and even though the detection threshold of our experiment was as low as 1% (Figure 3B and data not shown).

## Discussion

Here we present *in vitro* and *in vivo* data that address important questions regarding the potential use of drugs able to induce readthrough of stop codon mutations, to treat genetic disorders due to the presence of a PTC. Indeed, re-expression of a functional protein, which could provide a clinical benefit, is dependent on the correct realization of several steps. We first analyzed the efficacy of drug-mediated readthrough of premature termination codons by different compounds with antibiotic activities. We showed that negamycin, a dipeptide antibiotic, showed a higher suppression activity than gentamicin on several PTCs. In particular, the C1546X LAMA2 mutation that we analyzed in more detail is especially responsive to negamycin as compared to gentamicin and amikacin. This illustrates the importance of testing the efficiency of treatments in sensitive, rapid, and reproducible systems such as the one presented here. Since there are no hot spots of mutation within the LAMA2 gene, all mutations are 'private' and the consequences of the treatment may vary considerably between patients. The variable response to readthrough drugs has been recently illustrated in cystic fibrosis patients treated with gentamicin [30]. In that report, it was demonstrated, using the same dual reporter system as in the present work, that suppression of stop

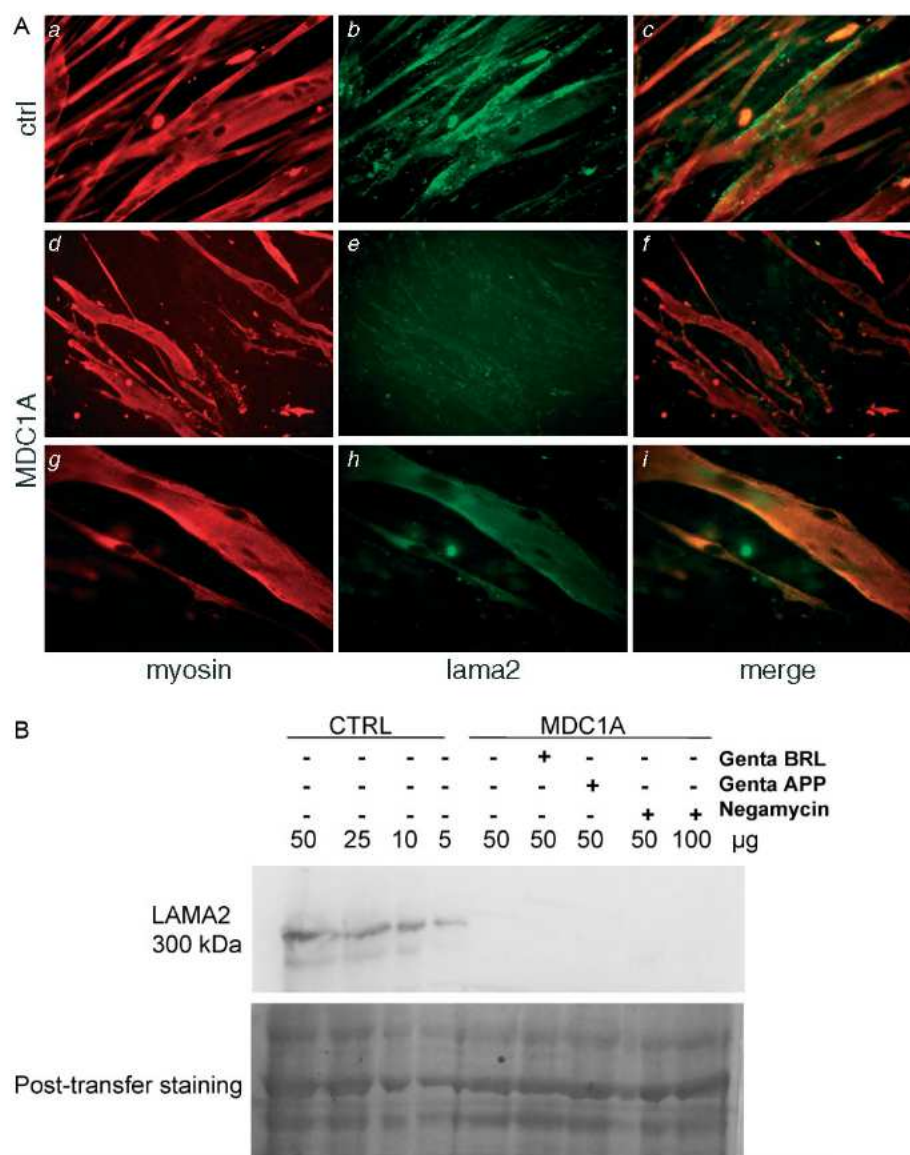
mutations in the CFTR gene with parenteral gentamicin can be predicted *in vitro*. The study established that in a small subgroup of patients with the Y122X mutation, gentamicin treatment was associated with clinical benefit and significant modification of the CFTR-mediated  $\text{Cl}^-$  transport in nasal and sweat gland epithelium [30].

Although readthrough-inducing compounds primarily affect translation, it is becoming clear that mutant mRNA stability is a significant factor in the process of termination readthrough. Importantly, we demonstrated that negamycin has a dual action since it enables readthrough levels high enough to strongly counteract mRNA degradation by the NMD pathway. Indeed, it has been shown that termination readthrough above the threshold value of 0.5% antagonizes NMD, leading to mRNA stabilization [46]. This likely explains the striking stabilization of LAMA2 mRNA observed following negamycin treatment, since we detected 3% of readthrough. On the other hand, the level of gentamicin-induced readthrough (0.6%) being just above the threshold value was probably not sufficient for mRNA stabilization. This would have important consequences for predicting the efficiency of pharmacological readthrough on patients' mutations before envisioning clinical trials, and again stresses the necessity to test *in vitro* the response of individual mutations to pharmacological treatment. Accordingly, gentamicin response was shown to depend on the efficiency of NMD in cystic fibrosis which varied greatly between patients and cell types [47]. In addition, in that study, the authors did not observe stabilization of the nonsense CFTR transcripts after gentamicin treatment in patients and cell lines [47], whereas it had been shown that G418, a more potent readthrough drug than gentamicin, could restore CFTR mRNA levels [16].

Recently, PTC124, a new molecule, has been shown to induce ribosomal readthrough of PTCs [48]. Interestingly, the levels of a nonsense-containing mRNA were unaffected in PTC124-treated cells. More generally, the synthesis and stability of few, if any, cellular mRNAs are altered in response to levels of PTC124. Although an off-target effect might be detrimental, the lack of stabilization of PTC-containing mRNAs by PTC124 may limit its use to mutations which do not lead to mRNA degradation by the NMD pathway.

In the present study, since gentamicin treatment did not allow stabilization of the mutant LAMA2 mRNA, it seems coherent that no re-expression of the protein occurred. On the other hand, considering the extent of mRNA stabilization that we detected following treatment with negamycin, the lack of laminin  $\alpha 2$ -chain re-expression may appear surprising. Several hypotheses, which are not mutually exclusive, may explain our results: (1) a longer treatment may be needed in order to be able to detect this large extracellular matrix protein; however, it was not possible to treat the cells for longer periods of time since the myotubes started detaching from the gelatine-coated dishes after 16 to 17 days of differentiation. (2) It is thought that PTCs would be misread by near-cognate aminoacyl-tRNAs bearing anti-codons with one





**Figure 3.** (A) Myosin and laminin  $\alpha$ 2-chain expression in control and MDC1A cultured myotubes after a 7-day negamycin treatment. Immunohistochemical analyses of primary myotubes from a control foetus (a–c) and a merosin-deficient CMD foetus (d–i) presenting a premature termination codon (C1546X) in the laminin  $\alpha$ 2 chain with antibodies against myosin (a, d, g) and the laminin  $\alpha$ 2 chain (b, e, h). Magnification 60 $\times$  (a–c, g–i). Magnification 25 $\times$  (d–f). c, f and i are merged images. (B) Western blot analysis in negamycin-treated and untreated control and MDC1A samples. Detection of laminin  $\alpha$ 2 chain on total proteins using an affinity purified polyclonal antibody (rabbit 180, a gift from Dr. Kevin P. Campbell). In order to control the amounts of proteins which had been loaded, the gel was stained with Coomassie blue following transfer

base difference. In *E. coli*, Nilsson and Ryden-Aulin [49] showed that stop codons UAG and UAA are replaced by glutamine whereas UGA would be substituted by tryptophan. Following this hypothesis, readthrough of the *LAMA2* UGA PTC would thus lead to the replacement of the normal cysteine by a tryptophan or an arginine residue. This residue is localized close to the junction site where the  $\alpha$ 2 chain of laminin-211 assembles with the  $\beta$ 1 and  $\gamma$ 1 chains to form the heterotrimeric molecule which will then be secreted. It seems likely that the insertion of a tryptophan residue would perturb this assembly and destabilize the protein which might thereafter be degraded. It should be noted that replacement of the cysteine residue by a tryptophan was predicted to be

deleterious by using the Web server PolyPhen [50] for polymorphism phenotyping [51]. It should be emphasized that treatments aiming at re-expressing a full-length protein by suppression of a PTC will be faced with this general problem. Although some structural proteins, like dystrophin, might tolerate large modifications of their coding sequences, enzymatic proteins, which are involved in most genetic disorders, will be very sensitive to the specific residue inserted in place of the PTC. This would necessitate a precise analysis of the site of the mutation in the context of the three-dimensional and functional structure of the protein, and of the potential near-cognate tRNA involved in decoding the stop codon upon drug treatment.

In conclusion, our study demonstrates that negamycin, a compound less toxic and more efficient for premature termination readthrough than gentamicin, appears an attractive alternative for treatment of patients carrying a PTC mutation. Its striking effect on mRNA stabilization suggests that, even for patients with a low level of mutant mRNA, treatment might be effective through readthrough-induced mRNA stabilization. However, although sufficient levels of mRNA are likely to be a preliminary requirement, correct synthesis of the corresponding full-length protein also depends on the effect of the insertion of a novel amino acid in place of the premature stop codon, which should be carefully considered prior to treatment.

Overall, our results emphasize that numerous steps need to be fulfilled to achieve a clinical benefit by inducing suppression of nonsense mutations.

## Acknowledgments

We gratefully acknowledge Gaëlle Piron (Inserm UMR-S787, Institut de Myologie, Paris, France) for expert technical advice for the primary cultures of myoblasts. We are grateful to Denis Furling (Inserm UMR-S787, Institut de Myologie, Paris, France) and Kevin P. Campbell (Molecular Physiology and Biophysics, Howard Hughes Medical Institute, Iowa City, USA) for the gift of antibodies MF20 and R180, respectively. We are grateful to Sèga N'Diaye (IGM, Université Paris-Sud, Orsay, France) for expert technical assistance with readthrough determination. VA was supported by a grant from the Fondation pour la Recherche Médicale. This work was funded by the Association Française contre les Myopathies (to VA and JPR), Association pour la Recherche sur le Cancer (grant N°3849 to JPR), INSERM and Université Paris 6 Pierre et Marie Curie, and the Japan Foundation for Aging and Health (to RM and PG). MPJ, GSB and VM are supported by CNRS. This work was supported by a Research Grant (18A-1, to RM) for Nervous and Mental Disorders from the Ministry of Health, Labor and Welfare and by a Grant for Research in Brain Science from the Ministry of Health, Labor and Welfare, Japan (to RM).

## References

- Fardeau M. Congenital myopathies. In *Skeletal Muscle Pathology*, Mastaglia FL, Detchant LWO (eds). Churchill Livingstone: Edinburgh, 1992; 237–281.
- Banker BQ. The congenital muscular dystrophies. In *Myology*, Engel AG, Franzini-Armstrong C (eds). McGraw-Hill: New York, 1994; 1275–1289.
- Tomé FMS, Guicheney P, Fardeau M. Congenital muscular dystrophies. In *Neuromuscular Disorders, Clinical and Molecular Genetics*, Emery AEH (ed). John Wiley: Chichester, 1998; 21–57.
- Tomé FM. The Peter Emil Becker Award lecture 1998. The saga of congenital muscular dystrophy. *Neuromuscul Disord* 1999; 30: 55–65.
- Helbling-Leclerc A, Zhang X, Topaloglu H, et al. Mutations in the laminin  $\alpha$ 2-chain gene (LAMA2) cause merosin-deficient congenital muscular dystrophy. *Nat Genet* 1995; 11: 216–218.
- Nissinen M, Helbling-Leclerc A, Zhang X, et al. Substitution of a conserved cysteine-996 in a cysteine-rich motif of the laminin  $\alpha$ 2-chain in congenital muscular dystrophy with partial deficiency of the protein. *Am J Hum Genet* 1996; 58: 1177–1184.
- Allamand V, Guicheney P. Merosin-deficient congenital muscular dystrophy, autosomal recessive (MDC1A, MIM#156225, LAMA2 gene coding for  $\alpha$ 2 chain of laminin). *Eur J Hum Genet* 2002; 10: 91–94.
- Allamand V, Sunada Y, Salih MAM, et al. Mild congenital muscular dystrophy in two patients with an internally deleted laminin  $\alpha$ 2-chain. *Hum Mol Genet* 1997; 6: 747–752.
- Guicheney P, Vignier N, Helbling-Leclerc A, et al. Genetics of laminin  $\alpha$ 2 chain (or merosin) deficient congenital muscular dystrophy: from identification of mutations to prenatal diagnosis. *Neuromuscul Disord* 1997; 7: 180–186.
- Palmer E, Wilhelm JM, Sherman F. Phenotypic suppression of nonsense mutants in yeast by aminoglycoside antibiotics. *Nature* 1979; 277: 148–150.
- Singh A, Ursic D, Davies J. Phenotypic suppression and misreading in *Saccharomyces cerevisiae*. *Nature* 1979; 277: 146–148.
- Burke JF, Mogg AE. Suppression of a nonsense mutation in mammalian cells in vivo by the aminoglycoside antibiotics G-418 and paromomycin. *Nucleic Acids Res* 1985; 13: 6265–6272.
- Martin R, Mogg AE, Heywood LA, Nitschke L, Burke JF. Aminoglycoside suppression at UAG, UAA and UGA codons in *Escherichia coli* and human tissue culture cells. *Mol Gen Genet* 1989; 217: 411–418.
- Kaufman RJ. Correction of genetic disease by making sense from nonsense. *J Clin Invest* 1999; 104: 367–368.
- Howard MT, Anderson CB, Fass U, et al. Readthrough of dystrophin stop codon mutations induced by aminoglycosides. *Ann Neurol* 2004; 55: 422–426.
- Bedwell DM, Kaenjak A, Benos DJ, et al. Suppression of a CFTR premature stop mutation in a bronchial epithelial cell line. *Nat Med* 1997; 3: 1280–1284.
- Barton-Davis ER, Cordier L, Shoturma DI, Leland SE, Sweeney HL. Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of *mdx* mice. *J Clin Invest* 1999; 104: 375–381.
- Wilschanski M, Famini C, Blau H, et al. A pilot study of the effect of gentamicin on nasal potential difference measurements in cystic fibrosis patients carrying stop mutations. *Am J Respir Crit Care Med* 2000; 161: 860–865.
- Clancy JP, Bebök Z, Ruiz F, et al. Evidence that systemic gentamicin suppresses premature stop mutations in patients with cystic fibrosis. *Am J Respir Crit Care Med* 2001; 163: 1683–1692.
- Keeling KM, Bedwell DM. Clinically relevant aminoglycosides can suppress disease-associated premature stop mutations in the *IDUA* and *P53* cDNAs in a mammalian translation system. *J Mol Med* 2002; 80: 367–376.
- Bidou L, Hatin I, Perez N, Allamand V, Panthier J-J, Rousset J-P. Premature stop codons involved in muscular dystrophies show a broad spectrum of readthrough efficiencies in response to gentamicin treatment. *Gene Ther* 2004; 11: 619–627.
- Kellermayer R, Szigeti R, Keeling KM, Bedekovics T, Bedwell DM. Aminoglycosides as potential pharmacogenetic agents in the treatment of Hailey-Hailey disease. *J Invest Dermatol* 2006; 126: 229–231.
- Wagner KR, Hamed S, Hadley DW, et al. Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutations. *Ann Neurol* 2001; 49: 706–711.
- Politano L, Nigro G, Nigro V, et al. Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results. *Acta Myologica* 2003; 22: 15–21.
- Wilschanski M, Yahav Y, Yaacov Y, et al. Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations. *N Engl J Med* 2003; 349: 1433–1441.
- James PD, Raut S, Rivard GE, et al. Aminoglycoside suppression of nonsense mutations in severe hemophilia. *Blood* 2005; 106: 3043–3048.
- Phillips-Jones M, Hill L, Atkinson J, Martin R. Context effects on misreading and suppression at UAG codons in human cells. *Mol Cell Biol* 1995; 15: 6593–6600.
- Howard MT, Shirts BH, Petros LM, Flanigan KM, Gesteland RF, Atkins JF. Sequence specificity of aminoglycoside-induced stop codon readthrough: potential implications for treatment of Duchenne muscular dystrophy. *Ann Neurol* 2000; 48: 164–169.
- Aurino S, Nigro V. Readthrough strategies for stop codons in Duchenne muscular dystrophy. *Acta Myologica* 2006; 25: 5–12.
- Sermet-Gaudelus I, Renouil M, Fajac A, et al. In vitro prediction of stop-codon suppression by intravenous gentamicin in patients with cystic fibrosis: a pilot study. *BMC Med* 2007; 5: 5.
- Swann SK. Aminoglycoside nephrotoxicity. *Semin Nephrol* 1997; 17: 27–33.
- Forge A, Schacht J. Aminoglycoside antibiotics. *Audiol Neurotol* 2000; 5: 3–22.



33. Dunant P, Walter MC, Karpati G, Lochmuller H. Gentamicin fails to increase dystrophin expression in dystrophin-deficient muscle. *Muscle Nerve* 2003; **27**: 624–627.
34. Wolstencroft EC, Mattis V, Bajer AA, Young PJ, Lorson CL. A non-sequence-specific requirement for SMN protein activity: the role of aminoglycosides in inducing elevated SMN protein levels. *Hum Mol Genet* 2005; **14**: 1199–1210.
35. Hamada M, Tadeuchi T, Kondo S, et al. A new antibiotic, negamycin. *J Antibiot* 1970; **23**: 170–171.
36. Uehara Y, Hori M, Umezawa H. Negamycin inhibits termination of protein synthesis directed by phage f2 RNA in vitro. *Biochim Biophys Acta* 1974; **374**: 82–95.
37. Arakawa M, Nakayama Y, Hara T, et al. Negamycin can restore dystrophin in mdx skeletal muscle. *Acta Myologica* 2001; **20**: 154–158.
38. Arakawa M, Shiozuka M, Nakayama Y, et al. Negamycin restores dystrophin expression in skeletal and cardiac muscles of mdx mice. *J Biochem (Tokyo)* 2003; **134**: 751–758.
39. Guicheney P, Vignier N, Zhang X, et al. PCR based mutation screening of the laminin alpha2 chain gene (LAMA2): application to prenatal diagnosis and search for founder effects in congenital muscular dystrophy. *J Med Genet* 1998; **35**: 211–217.
40. Bidou L, Stahl G, Hatin I, Namy O, Rousset J-P, Farabaugh PJ. Nonsense-mediated decay mutants do not affect programmed -1 frameshifting. *RNA* 2000; **6**: 952–961.
41. Martin R, Philips-Jones MK, Watson FJ, Hills LS. Codon context effects on nonsense suppression in human cells. *Biochem Soc Trans* 1993; **21**: 846–851.
42. Manuvakhova M, Keeling K, Bedwell DM. Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system. *RNA* 2000; **6**: 1044–1055.
43. Cassan M, Rousset JP. UAG readthrough in mammalian cells: Effect of upstream and downstream stop codon contexts reveal different signals. *BMC Mol Biol* 2001; **2**: 3.
44. Maquat LE. Nonsense-mediated mRNA decay in mammals. *J Cell Sci* 2005; **118**: 1773–1776.
45. Moriarty PM, Reddy CC, Maquat LE. Selenium deficiency reduces the abundance of mRNA for Se-dependent glutathione peroxidase 1 by a UGA-dependent mechanism likely to be nonsense codon-mediated decay of cytoplasmic mRNA. *Mol Cell Biol* 1998; **18**: 2932–2939.
46. Keeling KM, Lanier J, Du M, et al. Leaky termination at premature stop codons antagonize nonsense-mediated mRNA decay in *S. cerevisiae*. *RNA* 2004; **10**: 691–703.
47. Linde L, Boelz S, Nissim-Rafinia M, et al. Nonsense-mediated mRNA decay affects nonsense transcript levels and governs response of cystic fibrosis patients to gentamicin. *J Clin Invest* 2007; **117**: 683–692.
48. Welch EM, Barton ER, Zhuo J, et al. PTC124 targets genetic disorders caused by nonsense mutations. *Nature* 2007; **447**: 87–91.
49. Nilsson M, Ryden-Aulin M. Glutamine is incorporated at the nonsense codons UAG and UAA in a suppressor-free *Escherichia coli* strain. *Biochim Biophys Acta – Gene Structure and Expression* 2003; **1627**: 1–6.
50. Available: [www.bork.embl-heidelberg.de/PolyPhen](http://www.bork.embl-heidelberg.de/PolyPhen).
51. Sunyaev S, Ramensky V, Koch I, Lathe Wr, Kondrashov A, Bork P. Prediction of deleterious human alleles. *Hum Mol Genet* 2001; **10**: 591–597.