Gentamicin induces *LAMB3* nonsense mutation readthrough and restores functional laminin 332 in junctional epidermolysis bullosa

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Edited by James E. Cleaver, University of California, San Francisco, CA, and approved June 4, 2018 (received for review February 21, 2018)

Herlitz junctional epidermolysis bullosa (H-JEB) is an incurable, devastating, and mostly fatal inherited skin disease for which there is only supportive care. H-JEB is caused by loss-of-function mutations in *LAMA3*, *LAMB3*, or *LAMC2*, leading to complete loss of laminin 332, the major component of anchoring filaments, which mediate epidermal-dermal adherence. *LAMB3* (laminin β3) mutations account for 80% of patients with H-JEB, and ∼95% of H-JEB–associated *LAMB3* mutations are nonsense mutations leading to premature termination codons (PTCs). In this study, we evaluated the ability of gentamicin to induce PTC readthrough in H-JEB laminin β3-null keratinocytes transfected with expression vectors encoding eight different *LAMB3* nonsense mutations. We found that gentamicin induced PTC readthrough in all eight nonsense mutations tested. We next used lentiviral vectors to generate stably transduced H-JEB cells with the R635X and C290X nonsense mutations. Incubation of these cell lines with various concentrations of gentamicin resulted in the synthesis and secretion of full-length laminin β3 in a dose-dependent and sustained manner. Importantly, the gentamicin-induced laminin β3 led to the restoration of laminin 332 assembly, secretion, and deposition within the dermal/epidermal junction, as well as proper polarization of α6β4 integrin in basal keratinocytes, as assessed by immunoblot analysis, immunofluorescent microscopy, and an in vitro 3D skin equivalent model. Finally, newly restored laminin 332 corrected the abnormal cellular phenotype of H-JEB cells by reversing abnormal cell morphology, poor growth potential, poor cell-substratum adhesion, and hypermotility. Therefore, gentamicin may offer a therapy for H-JEB and other inherited skin diseases caused by PTC mutations.

gentamicin | epidermolysis bullosa | readthrough

Herlitz junctional epidermolysis bullosa (H-JEB) is a lethal skin-fragility disorder that occurs due to loss-of-function mutations in the *LAMA3*, *LAMB3*, or *LAMC2* gene, which encode laminin α3, β3, or γ2, respectively (1, 2). These monomers trimerize to form laminin 332, an essential component of structures called anchoring filaments (AFs). By binding to basal keratinocyte hemidesmosomes in the dermal/epidermal junction (DEJ), laminin 332 maintains adherence between the two layers of the skin (2). Loss of laminin 332 in patients who have H-JEB results in skin and mucocutaneous blistering, chronic infection, inadequate feeding, compromised wound healing, and refractory anemia (2, 3). Collectively, these derangements result in a 73% mortality rate, and few patients survive past 1 y of life, with death most commonly due to sepsis, failure to thrive, and respiratory failure (4–6). To date, there is no cure for H-JEB and therapeutic options are limited to palliative care (1, 5), despite various therapeutic strategies envisioned for JEB, including protein replacement therapy, bone marrow stem cell transplantation (SCT), and utilization of gene-corrected keratinocyte autografts (1, 7–11).

In ∼80% of all H-JEB cases, the *LAMB3* gene is affected (12). Although over 87 different mutations have been identified in H-JEB, ∼95% of disease-causing alleles contain *LAMB3* nonsense mutations that generate premature termination codons (PTCs), resulting in mRNA decay and synthesis of either no protein or a truncated protein incapable of forming functional laminin 332 (1, 12). Strikingly, in a recent review of 65 patients with H-JEB with known genotypes, the R635X nonsense mutation was detected in 84% of all patients with a mutated *LAMB3* gene (1). Thus, this mutational hotspot is a prime therapeutic target and warrants evaluation with nonsense mutation suppression therapy.

Aminoglycoside nonsense mutation suppression therapy using gentamicin has been shown to restore full-length, functional proteins in several genetic disorders, including cystic fibrosis (CF), Duchenne's muscular dystrophy (DMD), hemophilia, and retinitis pigmentosa (13–16), by mediating PTC readthrough via impaired codon/anticodon recognition after the aminoglycoside binds to mammalian ribosomal RNA (17, 18). Our recent work with recessive dystrophic epidermolysis bullosa (RDEB), a related mucocutaneous blistering disease caused by mutations in

Significance

Premature termination codons (PTCs) generated by nonsense mutations produce abnormal, short, or diminished proteins. Eighty-three percent of patients with Herlitz junctional epidermolysis bullosa (H-JEB), an inherited, incurable skin disease, harbor nonsense mutation(s) in genes encoding a structural protein (laminin 332) responsible for skin adherence. Gentamicin, a common antibiotic, was shown to induce readthrough of PTCs in various disease models. Using in vitro assays and 3D skin models, we found that H-JEB cells harboring nonsense mutations exposed to gentamicin produce full-length structural protein, deposit it correctly between skin layers, and exhibit reversal of other H-JEB–associated cellular abnormalities. Our findings indicate that gentamicin may present an immediate therapy for this otherwise fatal disease and other skin disorders caused by nonsense mutations.

Author contributions: J.C. and M.C. designed research; V.L., J.C., Y.H., M. Hirsch, M. Hao, and M.C. performed research; V.A., M.D.L., L.D.R., and J.W.B. contributed new reagents/analytic tools; J.C., Y.H., M. Hirsch, M. Hao, and M.C. analyzed data; and V.L., J.C., D.T.W., and M.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1803154115/-/DCSupplemental.

Published online June 26, 2018.

the gene encoding for type VII collagen (C7), demonstrated that gentamicin restored functional C7, which corrected dermal-epidermal separation, improved wound closure, and reduced blister formation in patients with RDEB with nonsense mutations (19). Moreover, there is already evidence that readthrough of H-JEB PTCs may lead to a much milder phenotype and improve clinical outcomes. Pacho et al. (20) showed that a patient with H-JEB with compound heterozygous nonsense mutations in the *LAMA3* gene (R943X/R1159X) unexpectedly improved with aging due to spontaneous readthrough of the R943X allele.

In this study, we tested the hypothesis that the aminoglycoside antibiotic gentamicin might have utility in the treatment of H-JEB caused by *LAMB3* nonsense mutations. We used site-directed mutagenesis to generate eight known H-JEB nonsense mutations and transfected these constructs into H-JEB laminin β3-null cells. Gentamicin treatment of these cells induced PTC readthrough and production of full-length laminin β3 protein to varying degrees in all eight nonsense constructs. Using an H-JEB laminin β3-null cell line engineered to express the R635X (H-JEB1) and C290X (H-JEB2) nonsense mutations, we show herein that gentamicin is able to induce PTC readthrough and restore functional laminin β3 protein, which successfully forms functional laminin 332 trimers. Gentamicin-restored laminin 332 in H-JEB1 and H-JEB2 keratinocytes reversed their abnormal cellular morphology, slow growth rate, poor cell-matrix adhesion, and hypermotility. Finally, in cultured human skin equivalents (SEs) composed of H-JEB1 and H-JEB2 keratinocytes, gentamicin treatment restored laminin 332 trimer, which incorporated into the DEJ. Our data demonstrate that gentamicin can induce PTC readthrough and restore functional laminin β3 protein and, ultimately, laminin 332 trimers in H-JEB cells with *LAMB3* nonsense mutations.

Results

Gentamicin Promotes Readthrough of Multiple H-JEB Nonsense Mutations and Induces Full-Length Laminin β3. Aminoglycoside-induced PTC readthrough efficacy is contingent on the type of aminoglycoside used and the specific sequence of the PTC generated by the nonsense mutation (18). To date, 20 different nonsense mutations have been reported in the *LAMB3* gene (3). To evaluate the potential general utility of gentamicin therapy for H-JEB–associated nonsense mutations, we used site-directed mutagenesis to introduce eight known H-JEB nonsense mutations into a *LAMB3* expression vector. Fig. 1*A* is a schematic of the laminin β3 molecule showing the location of each of the newly introduced nonsense mutations. We transfected these nonsense mutant constructs into a H-JEB laminin β3-null cell line, where the underlying defect is not caused by a nonsense mutation in *LAMB3*, and prepared cell lysates at 48 h after treatment with gentamicin. We used gentamicin concentrations of 500 μg/mL, which generated the strongest readthrough activity in the H-JEB cells without affecting cell viability (*SI Appendix*, Fig. S1). Gentamicin promoted PTC readthrough of every *LAMB3* nonsense mutant construct tested and resulted in production of full-length laminin β3 in all cases (Fig. 1*B*). As determined by densitometry analysis, gentamicin induced varying degrees of PTC readthrough, leading to laminin β3 expression between ~2% and ~27% (depending on the specific mutation) of that found in H-JEB cells transiently transfected with the wild-type *LAMB3* expression plasmid. Cells transiently transfected with the wild-type plasmid express laminin β3 at levels 90% of that seen in normal immortalized keratinocytes (IKCs) (Fig. 1*B*). Thus, our data demonstrate that all eight naturally occurring H-JEB nonsense mutations tested here are amenable to suppression by gentamicin in H-JEB cells, but with varying levels of efficacy.

Gentamicin Produces Full-Length Laminin β3 in a Dose-Dependent and Sustained Fashion. Next, we focused on the two nonsense mutations that exhibited the strongest responses in our transient transfection experiments for further functional studies: R635X and C290X. We used lentiviral expression vectors to stably express these two constructs in H-JEB laminin β3-null cells and established two cell lines: H-JEB/R635X (H-JEB1) and H-JEB/C290X (H-JEB2). As shown in Fig. 2*A*, gentamicin mediated a dose-dependent induction of full-length, 140-kDa laminin β3, while untreated cells displayed minimal laminin β3 expression. The optimal, nontoxic gentamicin concentration that maximized readthrough and full-length laminin β3 production was 500 μg/mL in both cell lines. Using this optimized concentration, quantification showed that gentamicin induced full-length laminin β3 expression in H-JEB1 and H-JEB2 to 11.78% and 30.74% of the levels observed in normal IKCs, respectively (Fig. 2*B*).

These immunoblot results were confirmed by immunofluorescence analysis. As shown in Fig. 2*C*, gentamicin-treated cells revealed positive staining for laminin β3 in both H-JEB1 and

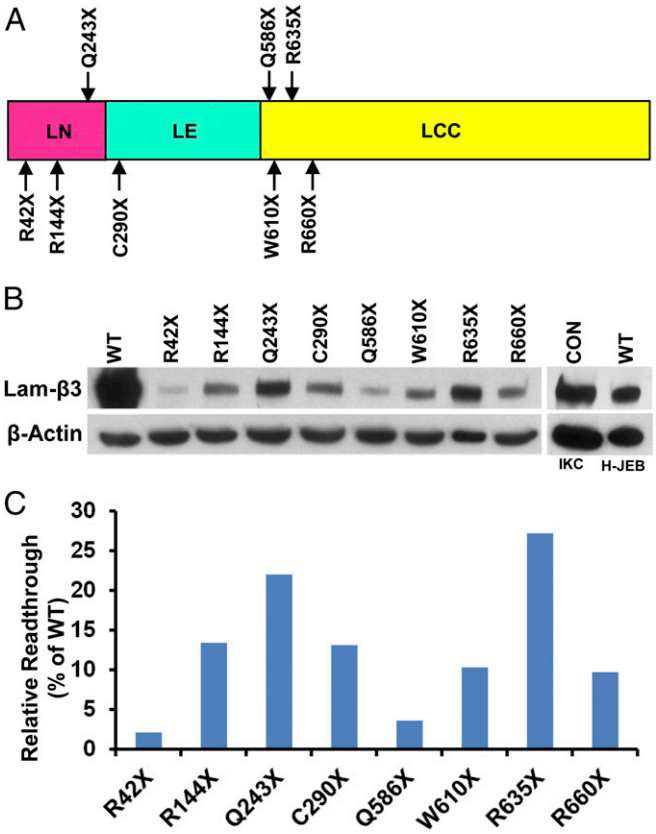

Fig. 1. Gentamicin restored full-length laminin β3 expression in laminin β3-null H-JEB keratinocytes transfected with multiple cDNA constructs coding for H-JEB nonsense mutations. (*A*) The 1,172-aa sequence of laminin β3 consists of a globular laminin N-terminal (LN) domain, laminin epidermal growth factor-like (LE) domains, and a laminin coiled-coil (LCC) domain. Eight *LAMB3* nonsense mutants, generated by site-directed mutagenesis, are shown in the schematic, with the approximate positions of the mutations indicated by arrows. (*B*) H-JEB keratinocytes that do not express laminin β3 were transfected with cDNA expression vectors coding for wild-type (WT) or various laminin β3 nonsense mutants, as indicated, for 48 h. Cells transfected with *LAMB3* nonsense mutations were then incubated with gentamicin (500 μg/mL) for an additional 48 h. Cell lysates were prepared and then subjected to 4–12% SDS/PAGE followed by immunoblot analysis using a monoclonal anti–laminin β3 antibody or an anti–β-actin antibody (loading control). (*Left*) First lane represents laminin β3 (Lam-β3) produced from H-JEB cells transfected with the expression vector for WT Lam-β3 (positive control). (*Right*) Comparison of Lam-β3 produced from nontransfected (CON) normal IKCs with H-JEB cells transfected with the WT expression plasmid. We performed three independent experiments, and similar results were obtained. (*C*) ImageJ analysis of laminin β3 expression normalized with β-actin from *B*. Results are displayed as a percentage of WT expression.

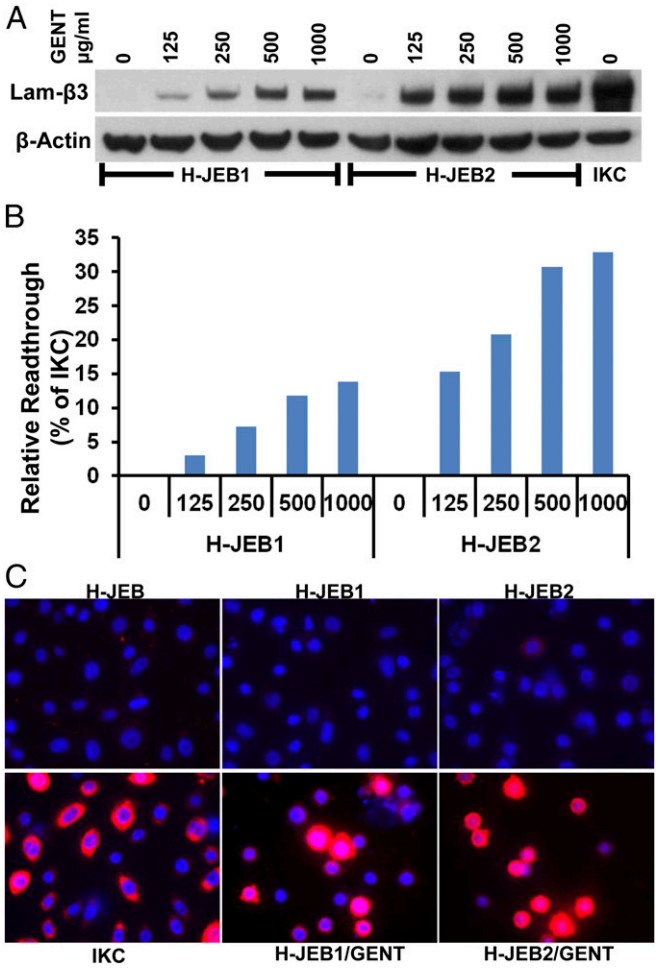

Fig. 2. Gentamicin mediates dose-dependent induction of full-length laminin β3 in laminin β3-null H-JEB keratinocytes stably transfected with the R635X and C290X nonsense mutation cDNA constructs. (*A*) Laminin β3 (Lam-β3)-null H-JEB cells were stably infected with Lam-β3 expression vectors coding for the R635X and C290X H-JEB–associated nonsense mutations, labeled H-JEB1 and H-JEB2, respectively. These cells were treated with increasing concentrations of gentamicin (GENT), as indicated, for 48 h. Cell lysates were prepared and then subjected to 4–12% SDS/PAGE, along with control lysates from normal IKCs, followed by immunoblot analysis with a monoclonal anti–Lam-β3 antibody or an anti–β-actin (loading control) antibody. GENT induced full-length Lam-β3 production in a dose-dependent manner in both mutant cell lines. Three independent experiments were performed with similar results. (*B*) ImageJ analysis of laminin β3 expression normalized with β-actin from *A*. Results are displayed as a percentage of IKC expression. (*C*) H-JEB1 and H-JEB2 keratinocytes were either untreated or treated with GENT (500 μg/mL) for 48 h and then subjected to immunofluorescence staining with a monoclonal anti-laminin β3 antibody. Note that GENT induced laminin β3 expression in both H-JEB1 and H-JEB2 keratinocytes. In contrast, untreated H-JEB1 and H-JEB2 keratinocytes lacked laminin β3 staining, similar to parental H-JEB cells. IKCs served as a positive control. Images (magnification, 40×) are representative of eight independent experiments.

H-JEB2, with 28% of H-JEB1 and 67% of H-JEB2 keratinocytes staining brightly (37% of IKCs stain brightly for laminin β3). In contrast, there was no detectable staining in either of the untreated cell groups. Taken together, these data indicate that gentamicin is capable of mediating PTC readthrough and inducing laminin β3 expression in both H-JEB1 and H-JEB2 cells.

Additionally, to better reflect clinical therapeutic use, we sought to determine the effects of continued dosing with gentamicin. To accomplish this, H-JEB1 and H-JEB2 cells were incubated with growth medium that received consecutive daily supplementation with gentamicin (500 μg/mL) for up to 5 d. Immunoblot analysis demonstrated that after 5 d of treatment, full-length laminin β3 expression increased up to 53.34% and 57.24% of the levels seen in normal keratinocytes for H-JEB1 and H-JEB2, respectively (Fig. 3 *A* and *B*). No apparent toxicity was observed with multiple treatments.

Since cells engineered to express exogenous nonsense mutations may respond differently than cells harboring the endogenous mutations would, we conducted experiments with primary JEB3 cells from a patient heterozygous for a nonsense mutation [1903C>T (R635X)] and an exonic splice site mutation (3009C-T) in *LAMB3* (21, 22). As shown in *SI Appendix*, Fig. S2, using optimal conditions (500 μg/mL and four daily doses), gentamicin treatment resulted in the production of full-length laminin β3 at levels 50.5% of those seen in normal human keratinocytes. In keeping with the previous report, there was minimal basal expression of laminin β3 in untreated cells (22). We did not observe any toxicity under these treatment conditions. Therefore, we conclude that gentamicin-induced readthrough of the endogenous R635X gene in JEB3 behaves similarly to the readthrough observed using the episomal vector expressing the R635X gene (H-JEB1). Given the difficulty in culturing these primary cells, all subsequent studies were conducted using our immortalized H-JEB cells engineered to stably express nonsense mutations.

We next evaluated how long laminin β3 production could be maintained after initial administration of gentamicin. As shown in Fig. 3*C*, full-length, 140-kDa laminin β3 was detected for up to 2 wk after initial treatment and levels were stable for both H-JEB1 and H-JEB2 keratinocytes. Secreted laminin β3 was also detectable for up to 2 wk in culture (Fig. 3*C*). Interestingly, additional experiments revealed that expression of laminin β3 was durable even 6 wk after initial administration (*SI Appendix*, Fig. S3). These data indicate that gentamicin-induced PTC readthrough and production of laminin β3 are sustained well after initial treatment.

After confirming gentamicin-mediated readthrough in our two stable H-JEB cell lines, we sought to test other known PTC readthrough agents and compare their efficacy with gentamicin. These agents include aminoglycosides, such as paromomycin and amikacin (23); PTC124 (a nonaminoglycoside PTC readthrough compound) (24); and amlexanox (an immunomodulatory drug that was recently reported to induce PTC readthrough via the inhibition of nonsense-mediated decay of RNA in human cells with nonsense mutations, including cells originating from patients with RDEB) (25). Using concentrations from previous studies that did not affect cell viability (23, 25–27) (*SI Appendix*, Fig. S1), none of these compounds induced PTC readthrough and detectable laminin β3 expression above basal levels in either H-JEB1 or H-JEB2 (Fig. 4). To confirm these results, we tested amlexanox at the highest concentrations reported in the literature in all eight H-JEB nonsense mutation constructs transfected into H-JEB cells. A marginal level of laminin β3 was detected by immunoblot analysis of cell lysates with the Q243X mutation, but no protein was detected with the other seven mutations (*SI Appendix*, Fig. S4).

Gentamicin-Induced Laminin β3 Assembles into Laminin 332 Trimers That Deposit into the Extracellular Space. Laminin β3 associates with the α3 and γ2 chains to form functional laminin 332 heterotrimers that are secreted into the medium in normal cultured keratinocytes. To determine if gentamicin-induced laminin β3 can contribute to the assembly of mature laminin 332 trimers, conditioned media from the gentamicin-treated H-JEB1 and H-JEB2 cells were collected after 72 h, concentrated, and then subjected to immunoblot analysis with a polyclonal anti-laminin 332 antibody. As shown in Fig. 5*A*, 440-kDa and 400-kDa doublets (corresponding to the processed and cleaved laminin 332 trimers, respectively, postsecretion) (28) were detected in

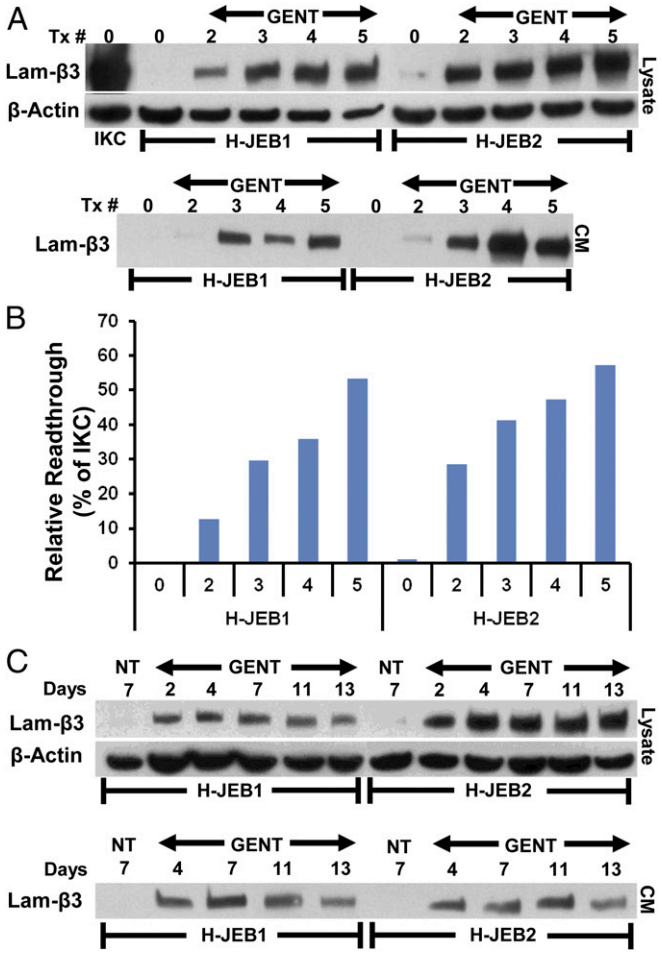

Fig. 3. Gentamicin-induced production of full-length laminin β3 is sustained and increases with continued dosing in H-JEB keratinocytes stably transfected with the R635X and C290X nonsense mutation cDNA constructs. (*A*) H-JEB1 and H-JEB2 keratinocytes were incubated with growth medium in the absence of gentamicin (GENT) or were given consecutive daily treatments (Tx) of GENT (500 μg/mL) for up to 5 d. Cell lysates were prepared and then subjected to 4–12% SDS/PAGE, followed by immunoblot analysis with a monoclonal anti-laminin β3 (Lam-β3) antibody or an anti–β-actin (loading control) antibody. Note that GENT-induced, full-length Lam-β3 expression increased with daily treatment. We performed three independent experiments with similar results. (*B*) ImageJ analysis of laminin β3 expression normalized with β-actin in lysates from *A*. Results are displayed as a percentage of IKC expression. (*C*) H-JEB1 and H-JEB2 keratinocytes were incubated with growth medium in the absence or presence of GENT (500 μg/mL) for 48 h, which were then changed to normal growth medium for the duration of the experiment. Cell lysates (Lysate) and conditioned media (CM) were prepared at various days after treatment, as indicated, and then subjected to 4–12% SDS/PAGE followed by immunoblot analysis with a monoclonal anti–Lam-β3 antibody or an anti–β-actin (loading control). Lysate and CM from day 7 untreated cells (NT) were run as negative controls. Note that GENT induced sustained Lam-β3 production that was readily detectable 2 wk after initial treatment. We performed three independent experiments with similar results.

the media from gentamicin-treated H-JEB1 and H-JEB2, respectively, similar to those seen in normal human keratinocytes. In contrast, no trimer was detected in the media of the untreated cells. These findings suggest that the gentamicin-induced laminin β3 is capable of forming trimers that are properly cleaved to mature laminin 332 in the extracellular space.

Immunoblot results were further confirmed by immunofluorescent analysis using a polyclonal antibody recognizing native laminin 332. As shown in Fig. 5*B*, without gentamicin treatment, there is virtually no secreted laminin 332 trimer detectable from H-JEB1 and H-JEB2 cells when probed with anti-laminin 332 antibody. In contrast, immunostaining of gentamicin-treated cells displayed not only increased intracellular laminin expression but also deposition of laminin 332 into the surrounding extracellular matrix (ECM), although the amount of deposited laminin 332 was significantly lower than that of normal keratinocytes. Taken together, these data suggest that gentamicin-induced laminin β3 contributes to the formation of mature laminin 332.

Gentamicin Normalizes the Morphology, Proliferation Rates, Poor Cell-Matrix Adhesion, and Hypermotility of H-JEB Cells. Gentamicin's suppressive effects on PTCs are mediated by a mispairing between the stop codon and near-cognate aminoacyl tRNA, resulting in insertion of a random amino acid instead of chain termination (17, 18). Since the amino acid inserted at the PTC may differ from the residue originally residing in wild-type laminin β3, the readthrough product may have impaired functionality compared with the normal wild-type protein. For this reason, we evaluated whether the laminin 332 produced after gentamicin treatment of H-JEB cells is actually functional. It has previously been shown that JEB cells display abnormal cellular phenotypes, including abnormal morphology, poor growth potential, and reduced cell adhesion (29). As shown in Fig. 6*A*, under light microscopy, we observed that ∼66% of keratinocytes cultured from the H-JEB parent cells are polymorphic and show multiple morphological abnormalities, including unusual, elongated, spindle-shaped cells as well as rounder cells with enlarged cytoplasm, compared with keratinocytes cultured from normal controls (2% abnormal). In contrast, treatment with gentamicin reduced the high percentage of cells with irregular morphology in untreated H-JEB1 and H-JEB2 cells to that approaching normal keratinocytes (4% and 6% abnormal, respectively). Untreated H-JEB1 and H-JEB2 keratinocytes maintained all of the morphological abnormalities of the H-JEB parent cells. These findings suggest that gentamicin treatment reverses the abnormal morphology of H-JEB cells.

We next determined if gentamicin-induced laminin 332 could correct the poor growth potential of H-JEB cells. As shown in Fig. 6*B*, compared with normal keratinocytes, H-JEB parent cells demonstrated a significantly reduced growth rate, as previously described (29). In contrast, gentamicin treatment of H-JEB1 and H-JEB2 keratinocytes enhanced their growth potential to levels approaching normal keratinocytes.

Additionally, we evaluated whether treatment with gentamicin can enhance the faulty cell-substratum adhesion of H-JEB cells. We subjected normal keratinocytes, as well as H-JEB1 and H-JEB2 keratinocytes (either untreated or treated with gentamicin), to a well-established kinetic cell-detachment assay. Fig. 6*C*

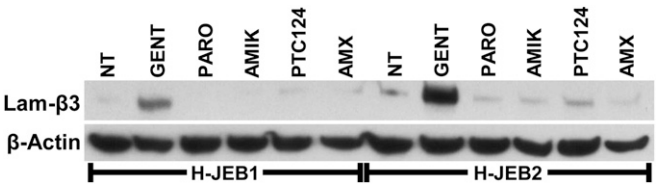

Fig. 4. Efficacy of various readthrough compounds in inducing laminin β3 (Lam-β3) production. H-JEB1 and H-JEB2 keratinocytes were untreated (NT) or treated with gentamicin (GENT; 500 μg/mL), paromomycin (PARO; 2,000 μg/mL), amikacin (AMIK; 1,000 μg/mL), PTC124 (20 μg/mL), and amlexanox (AMX; 7.5 μg/mL), as indicated, for 48 h. Cell lysates were prepared and then subjected to 4–12% SDS/PAGE, followed by immunoblot analysis with a monoclonal anti–Lam-β3 antibody or an anti–β-actin (loading control). Note that no drug except GENT promoted significant readthrough and production of Lam-β3 over basal levels. We performed three independent experiments, and similar results were obtained.

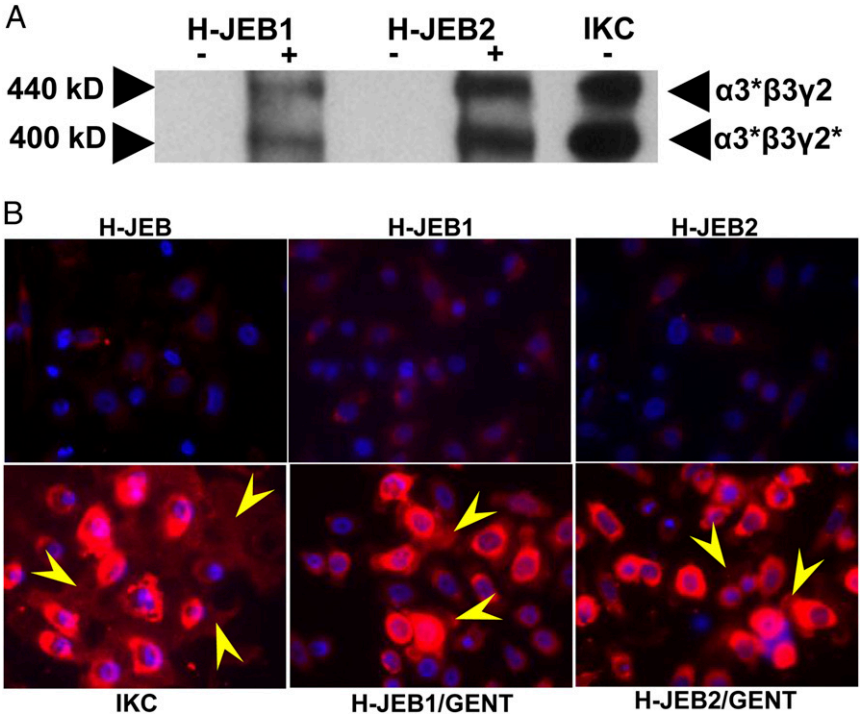

Fig. 5. Gentamicin-induced laminin β3 organizes into functional laminin 332 trimers that deposit into the extracellular space. (*A*) H-JEB1 and H-JEB2 keratinocytes were either untreated (–) or treated (+) with gentamicin (500 μg/mL) for 72 h. Concentrated conditioned media were prepared and then subjected to 4–12% SDS/PAGE followed by immunoblot analysis using a polyclonal anti-laminin 332 antibody. Concentrated conditioned medium from IKC cells was run as a positive control. α3*β3γ and α3*β3γ* represent cleaved laminin 332 trimers after extracellular processing. Note that laminin 332 trimers (of 440 kDa and 400 kDa) were detected in the media of H-JEB1 and H-JEB2 cells treated with gentamicin, which were absent in untreated samples. (*B*) H-JEB1 and H-JEB2 keratinocytes were either untreated or treated with gentamicin (GENT; 500 μg/mL) for 48 h and then subjected to immunofluorescence staining with a polyclonal anti-laminin 332(β3) antibody. Note that GENT induced laminin 332 deposition, which was detected in the ECM (yellow arrowheads) in both H-JEB1 and H-JEB2 cells. In contrast, untreated H-JEB cells lacked laminin 332 expression. H-JEB keratinocytes were included as an additional negative control, and normal IKC cells were used as a positive control. Images (magnification, 40×) are representative of six independent experiments.

depicts the number of cells detached from the substratum 5 min after the addition of trypsin, expressed as a percentage of the total number of cells for each cell type and treatment. Untreated H-JEB cells exhibited poor cell-substratum adhesion, with over 80% of cells detaching at 5 min compared with normal keratinocytes, which exhibited less than 5% detachment. Conversely, gentamicin-induced laminin 332 in the treated H-JEB cells enhanced their cell-matrix adhesion strength to a degree similar to that seen in normal keratinocytes. These data indicate that gentamicin-induced functional laminin 332 can correct the poor substrate adhesion associated with H-JEB cells.

We have previously shown that RDEB cells demonstrated hypermotility compared with normal human keratinocytes (30). To determine if H-JEB cells also exhibit the same phenotype and, if so, whether gentamicin-induced laminin 332 can reverse their hypermotility, we subjected normal, parental H-JEB, and H-JEB1 and H-JEB2 keratinocytes, either untreated or treated with gentamicin, to a well-established keratinocyte migration assay. Fig. 6*D*, *Top* shows representative microscopic fields of these cells. Untreated H-JEB1 and H-JEB2 keratinocytes exhibited cellular hypermotility that was identical to laminin β3-null H-JEB parent cells. We measured cellular motility using a published, computer-assisted migration index (MI), which is calculated as the percentage of the microscopic field consumed by motility tracks (30) (Fig. 6*D*, *Bottom*). Interestingly, H-JEB cells displayed enhanced motility with MIs of 35.6 in comparison to MIs of 23.2 for normal human keratinocytes. In contrast, gentamicin-treated H-JEB1 and H-JEB2 keratinocytes exhibited normalized cellular motility with MIs of 24.07 and 22.92, respectively, similar to normal human keratinocytes. Therefore, gentamicin effectively promoted PTC readthrough and produced functional laminin 332 that corrected the hypermotility of H-JEB cells.

Gentamicin-Induced Laminin 332 Trimers Localize to the DEJ of H-JEB SEs. After achieving restoration of laminin 332 in H-JEB1 and H-JEB2 keratinocytes and demonstrating correction of H-JEB–associated abnormal cellular phenotypes, we sought to determine if gentamicin-induced laminin 332 could incorporate into the DEJ. To test this, we used an in vitro 3D SE model, where an acellular collagen gel was infused with normal human fibroblasts and then seeded with keratinocytes, as described previously (31). In this series of experiments, the SEs were constructed with normal keratinocytes or with H-JEB1 or H-JEB2 keratinocytes, and they were either treated with gentamicin or untreated. Up to 2 wk after the SEs were established in culture, they were subjected to hematoxylin and eosin staining (*SI Appendix*, Fig. S5) and immunofluorescence staining using a polyclonal anti-laminin 332(β) antibody that recognizes the β3 chain. As seen in the first row of Fig. 7*A*, there was no detectable laminin 332 deposition in SEs generated from untreated H-JEB1 and H-JEB2 cells. In contrast, there was strong linear staining at the DEJ between the keratinocytes and the dermal equivalent in the SEs generated from gentamicin-treated H-JEB1 and H-JEB2 cells. This pattern was similar to SEs composed of normal human keratinocytes and fibroblasts. To confirm if this linear deposition was actually within the DEJ, the SE sections were colabeled with a monoclonal anti-laminin 332(α) antibody along with either anti-α6 integrin or anti-β4 integrin antibodies targeting the α6β4 integrin, an adhesion molecule located in basal keratinocytes that acts as the nucleating center for hemidesmosome

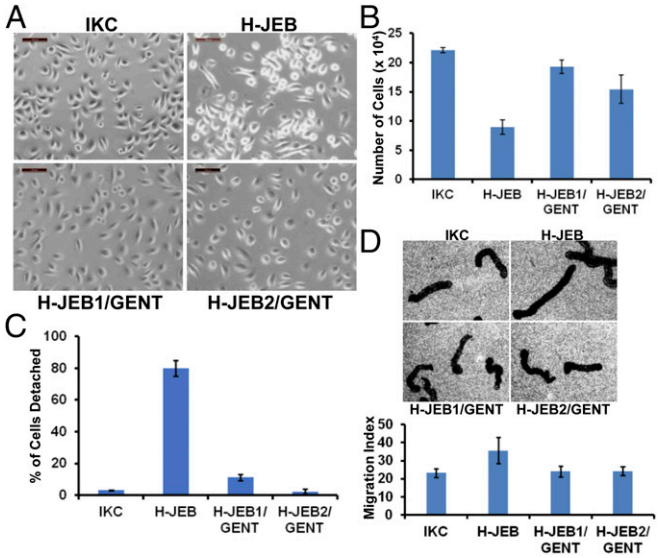

Fig. 6. Gentamicin normalizes the cell morphology, cellular proliferation, substratum attachment, and hypermotility of H-JEB cells. (*A*) Under light microscopy, the morphology of H-JEB keratinocytes is corrected by gentamicin (GENT). (*Top Left*) Cultured normal IKCs show a compact, typical epithelial cell morphology. (*Top Right*) H-JEB keratinocytes, in contrast, exhibit a mixture of cell morphologies, with some cells displaying an elongated, spindle-shaped morphology, while other cells have a rounder shape with enlarged cytoplasm. Note that many cells also appear poorly attached to the culture plate. (*Bottom Left* and *Bottom Right*) H-JEB1 and H-JEB2 keratinocytes treated with GENT display a more typical epithelial cell morphology reminiscent of normal human keratinocytes. (Scale bars: 100 μm.) (*B*) IKCs, laminin β3-null H-JEB keratinocytes, and GENT-treated H-JEB1 and H-JEB2 keratinocytes were seeded to plates with an initial cell count of 75,000. Keratinocytes were then incubated in growth medium for 48 h and supplemented with GENT (500 μg/mL) where indicated. After 48 h, the plates were trypsinized and the cells were counted. (*C*) IKCs, laminin β3-null H-JEB keratinocytes, H-JEB1 keratinocytes, and H-JEB2 keratinocytes were seeded onto tissue culture plates. The H-JEB1 and H-JEB2 keratinocytes were treated with GENT (500 μg/mL) for 48 h. All cells were simultaneously trypsinized, and the number of cells detached after 5 min was determined and expressed as a percentage of the total number of cells for each cell line. After 5 min, H-JEB cells were nearly completely detached, while IKCs and treated H-JEB1 and H-JEB2 keratinocytes exhibited less than 10% detachment. Each value is the average of triplicates from three independent experiments. (*D*) H-JEB keratinocytes were either untreated or treated with GENT (500 μg/mL) for 48 h and then subjected, along with IKCs and laminin β3-null H-JEB parent keratinocytes, to a colloidal gold salt migration assay using collagen I as a matrix. (*Top*) Representative fields photographed at a magnification of 40× under dark-field optics. (*Bottom*) Computer-generated migration index is the percentage of the total field area occupied by migration tracks. Error bars indicate SEs of three different experiments. Note that H-JEB keratinocytes were hypermotile compared with IKCs. In contrast, GENT-treated H-JEB1 and H-JEB2 cells demonstrated reduced motility similar to the motility of IKCs. Untreated H-JEB1 and H-JEB2 keratinocytes exhibited cellular hypermotility that was identical to laminin β3-null H-JEB parent cells.

formation in normal skin (32). Coimmunolabeling of SEs composed of H-JEB1 and H-JEB2 cells treated with gentamicin revealed strong linear staining of both integrins and laminin 332 colocalized at the DEJ between the keratinocytes and the dermal equivalent. In contrast, SEs generated from untreated H-JEB1 and H-JEB2 cells displayed only intracellular staining, and no linear staining, at the DEJ. It is possible that the formation of laminin 332 trimer is a prerequisite for correct localization of α6β4 integrin to the DEJ. Quantitation with ImageJ (NIH) analysis showed that the level of laminin 332 deposited at the DEJ in SEs composed of gentamicin-treated H-JEB1 and H-JEB2 keratinocytes was 74.5% and 78.4%, respectively, of the level of laminin 332 produced from SEs composed of normal keratinocytes. We conclude that gentamicin-induced laminin 332 is capable of incorporating into the DEJ in vitro.

Discussion

In this study, we tested eight known nonsense mutations affecting *LAMB3* and found that gentamicin promoted varying degrees of PTC readthrough in H-JEB keratinocytes transfected with these mutations. Two mutations were selected for further functional studies due to prevalence (R635X) and location on the ECM-binding domain of laminin β3 (C290X). We further demonstrated that gentamicin promoted PTC readthrough in H-JEB cells harboring these two mutations, ultimately resulting in production of functional laminin 332 that corrects H-JEB–associated abnormal cellular phenotypes and deposits into the DEJ of SEs. There are at least 20 reported unique *LAMB3* nonsense mutations associated with H-JEB; thus, our data may not reflect all potential responses in patients harboring other types of nonsense mutations. It should be emphasized, however, that three nonsense mutations investigated here, R42X, Q243X, and R635X, are recurrent mutations in patients with H-JEB and account for ∼60% of all patients with H-JEB and 74% of patients with H-JEB who have nonsense mutations (1).

It has been reported that the type of stop codon (in order of propensity for readthrough: UGA, UAG, UAA) and the nearby nucleotide sequence context surrounding each stop codon (i.e., the immediate upstream −1 nt and downstream +4 nt) are major factors in determining readthrough efficacy for a given PTC. In particular, the nucleotide C or U at the −1 position and the nucleotide C at the +4 position flanking the PTC promote stronger readthrough efficiency compared with other nucleotides at those respective positions (20, 33, 34). Although we did not perform a systematic analysis where artificial mutations were created in the local nucleotide context of each H-JEB PTC, as described elsewhere (20, 33, 34), our data support the importance of the −1 and +4 positions in determining the readthrough efficacy in seemingly identical H-JEB stop codons. The stop codons and nucleotide context of each of the eight H-JEB–associated PTCs (six UGA and two UAG) analyzed in this study are summarized in *SI Appendix*, Table S1. In examining the two UAG stop codons, the presence of a C in the +4 position of Q243X is the only difference that may offer an explanation for its order-of-magnitude greater response to gentamicin compared with Q586X. Also, although none of the six mutations bearing the UGA stop codon (R42X, R144X, C290X, W610X, R635X, and R660X) have the consensus optimal C in the +4 position, the strongest responders tended to have either a C or U in the −1 position (R635X and C290X), both of which are predicted to be the preferred nucleotides for readthrough (20, 34). It should be pointed out, however, that the limited number of mutations analyzed here lacks the necessary statistical power to make any definitive conclusions regarding the relationship between readthrough efficacy and the type of PTC or surrounding nucleotides.

The ultimate goal of any H-JEB therapy is to generate adequate levels of functional laminin 332 that properly localizes to the DEJ of the patients' skin and provides sufficient adherence between the epidermis and dermis. In our studies of RDEB, we observed that gentamicin induced PTC readthrough and restored C7 at the DEJ to levels more than 50% of normal human skin. Several lines of evidence show that only 35% of normal is sufficient to provide good epidermal-dermal adherence in RDEB (35, 36). Although the minimum amount of laminin 332 secreted by cells required to restore epidermal-dermal adherence is not known, a study analyzing keratinocytes and skin biopsies collected from a patient with H-JEB with spontaneous *LAMA3* PTC readthrough provides some insight (20). Although this patient's keratinocytes secreted significantly less laminin α3 than healthy controls in cell culture, he continued to experience clinical improvement, with laminin 332 levels in his DEJ

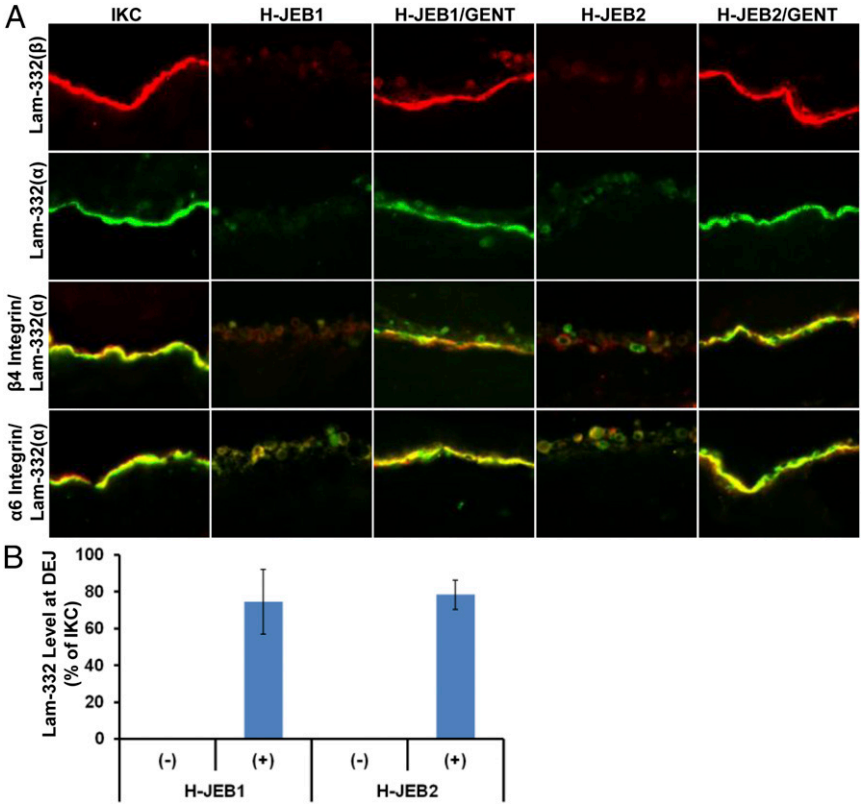

Fig. 7. Gentamicin-induced laminin 332 incorporates into the DEJ of in vitro SEs. (*A*) Cryosections from 1-wk-old SEs were subjected to immunofluorescent labeling using a polyclonal anti-laminin 332(β3) antibody [Lam-332(β); row 1] and a monoclonal anti-laminin 332(α3) antibody [Lam-332(α), row 2], and were then colabeled with antibodies to either the α6 integrin chain (α6 Integrin, row 3) or the β4 integrin chain (β4 Integrin, row 4). From left to right, the columns represent SEs established from normal IKCs and untreated and treated (500 μg/mL gentamicin; GENT) H-JEB1 and H-JEB2 cells. Results are representative images from triplicate SEs. Note that the antibodies to both the α6 integrin and β4 integrin colocalize with the labeling of the Lam-332(α) antibody. (*B*) Mean average fluorescence intensity of laminin 332 (Lam-332) immunoreactivity was obtained from representative images from three independent SEs. Frozen sections were probed with a polyclonal laminin 332(β3) antibody, and the intensity of Lam-332 at the DEJ of each specimen was measured by computer-assisted ImageJ software and compared with the intensity of Lam-332 in SEs derived from IKCs. Values represent the intensity of Lam-332 in the DEJ of the SEs expressed as a percentage of the average intensity obtained from IKCs (100%). Data represent the mean ± SD.

eventually approaching those of a normal control. This suggests that, although not precisely quantified, the amount of laminin 332 secreted from keratinocytes required to restore normal expression at the DEJ and maintain dermal-epidermal adherence may be significantly less than that produced by normal keratinocytes. In the present study, we observed that two administrations of gentamicin induced PTC readthrough in H-JEB1 and H-JEB2 cells to levels of 12.76% and 28.46% of those seen in normal cells, respectively. Most importantly, after 5 d of daily gentamicin dosing in both H-JEB keratinocytes, the levels of laminin β3 increased to more than 50% of those seen in normal keratinocytes. Additionally, even without continued dosing, the level of full-length laminin β3 was maintained without diminishing for up to 4 wk after the initial two treatments. Given this robust response to short-term gentamicin treatment and the durability of laminin β3 detected in media and cell lysates, it is possible that short-term, pulsed gentamicin therapy might be sufficient to provide enough laminin 332 to improve the clinical phenotype of a patient with H-JEB.

Alternative therapeutic approaches to treat JEB are currently being explored, including allogenic SCT and autologous transgenic skin graft therapy. Allogenic SCT via bone marrow or peripheral blood harvesting has advanced to clinical trials, but a retrospective study evaluating the treatment of infants with severe H-JEB found only limited short-term efficacy in two patients before they died (1). In a recent study by Hirsch et al. (10), autologous gene-corrected, transgenic keratinocytes were grafted onto large open wounds of a 7-y-old child. The grafted skin remained closed, resisted mechanical stress, and continued to express laminin 332 for at least 21 mo. While promising, a less expensive treatment, such as topical or systemic gentamicin, might benefit patients with H-JEB who have nonsense mutations, while also being readily available. In addition, gentamicin applied topically or systemically would not expose the patient to live cells or viral vectors and would not involve surgical procedures.

Gentamicin is a well-characterized antibiotic that is approved by the US Food and Drug Administration for infections, and its potential side effects (ototoxicity and/or renal impairment) can be easily monitored and prevented. Moreover, in patients with RDEB who have nonsense mutations, we recently demonstrated that topical and intradermal gentamicin proved to be both safe and effective (19). Nonetheless, other nonaminoglycoside derivatives with potentially greater PTC readthrough or fewer side effects than gentamicin have been reported, two of which are PTC124 (ataluren) and amlexanox (25, 37, 38). While PTC124 showed promise in preclinical experiments for CF and DMD, subsequent clinical trials in patients with CF and DMD, as well as the in vitro results from this study and data from other disease models, including RDEB, demonstrated limited or no ability of PTC124 to induce PTC readthrough (26, 37–43). Amlexanox has recently been shown to enhance PTC readthrough and restore C7 expression in some RDEB cells (25). However, in our present study, we found that amlexanox was only able to induce laminin

β3 expression in one of eight H-JEB–transfected nonsense mutations. Additionally, we failed to detect readthrough activity using two other aminoglycosides: amikacin and paromomycin. This is consistent with other previously published reports (26, 44–46).

In summary, this study demonstrates that gentamicin-induced suppression of H-JEB PTC mutations restores laminin β3 expression and formation of functional laminin 332 that incorporates into the DEJ. The future goals of our research involve advancing gentamicin into human clinical trials and identifying more advantageous modes of delivery. The many disparate organ systems affected by H-JEB make i.v. infusion an ideal route for administering gentamicin, but topical administration and nebulized administration are alternative therapies that present less systemic exposure while targeting the skin and lungs, two critical organ systems whose pathological involvement is associated with H-JEB's high infant mortality (16, 19, 47). Ultimately, gentamicin therapy may hold great promise for the 80% of patients with H-JEB who harbor nonsense mutations, as well as for suppressing PTCs in other inherited skin conditions due to nonsense mutations.

Materials and Methods

Cell Lines and Culture Conditions. The human embryonic kidney cell line 293T (American Type Culture Collection) was cultured in Dulbecco's modified essential medium (DMEM) supplemented with 10% FBS. An immortalized H-JEB keratinocyte cell line harboring a laminin β3-null mutation was cultured in serum-free keratinocyte growth medium supplemented with human keratinocyte growth supplement (HKGS) (Epilife; GIBCO BRL) (48, 49). Primary human keratinocytes were purchased from Thermo Fisher Scientific. Primary JEB3 keratinocytes from a patient heterozygous for a nonsense mutation [1903C>T (R635X)] and an exonic splice site mutation (3009C-T) in *LAMB3* were obtained with the approval of the University Hospital of the Paracelsus Medical University, Salzburg after written informed consent was given by the patient, as described previously (21, 22). Normal human or H-JEB keratinocytes were immortalized using the E6 and E7 genes of human papillomavirus type 6 as described elsewhere (50). All H-JEB cells, both primary and immortalized, were plated and cultured on collagen 1-coated plates and grown with Epilife, as described above. Adult normal primary dermal fibroblasts were purchased from Thermofisher Scientific and cultured in DMEM/Ham's F-12 (1:1) supplemented with 10% FBS. Primary fibroblasts were passaged as they reached confluence, and all SE experiments were performed using fibroblasts between passages 4 and 6.

Site-Directed Mutagenesis, Cell Transfection, and Cell Transduction. Site-directed mutagenesis was performed on *LAMB3* cDNA in the pRC/CMV vector using a commercial kit (QuikChange II Site-Directed Mutagenesis Kit; Stratagene, Inc.) according to the manufacturer's instructions, as described previously (30). Briefly, a pair of complementary primers with 39 bases was designed, and a mutation to change specific amino acid residues to a stop codon was placed in the middle. We generated primers for the following eight mutations associated with H-JEB reported in the literature: R42X, R144X, Q243X, C290X, Q586X, W610X, R635X, and R660X. Parental cDNA inserted in pRC/CMV was amplified using *Pyrococcus furiosus* DNA polymerase with these primers for 16 cycles in a DNA thermal cycler. After digestion of the parental DNA with DpnI, the amplified DNA with the nucleotide substitutions incorporated was transformed into *Escherichia coli* (XL1-Blue). The mutations were confirmed by automated DNA sequencing.

The expression vector encoding wild-type or mutant *LAMB3* cDNA was used to transfect H-JEB cells using Lipofectamine LTX with Plus Reagent (Life Technologies). Cells were plated and transfected according to the manufacturer's instructions. Twenty-four hours after transfection, the medium was changed to fresh growth medium containing gentamicin for 48 h.

To further investigate the short- and long-term effects of gentamicin and other readthrough compounds on laminin β3-null H-JEB cells harboring the R635X and C290X nonsense mutations, we stably transduced *LAMB3* expression vectors for those mutations into parent H-JEB laminin β3-null cells and established the H-JEB1 and H-JEB2 cell lines, respectively. Briefly, DNA containing the entire *LAMB3* coding sequence was cloned into the pRRLsinhCMVGFPpre viral expression vector used previously (30). After confirmation of correct incorporation with automated DNA sequencing, vectors were transfected along with pΔR and VSV-G packaging vectors into 293T cells. Active virus was harvested and supplemented to H-JEB cells, with an overall infection rate of greater than 90% for each construct.

Drug Treatment and Immunoblotting. In experiments where an aminoglycoside or PTC124 (Selleckchem) was used to induce PTC readthrough, normal human keratinocytes or H-JEB cells were transiently transfected with cDNA expression constructs containing nonsense mutations at 60–70% confluence. Twenty-four hours after transfection, the media were replaced and supplemented with or without gentamicin (Sigma), paromomycin (Sigma), amikacin (Sigma), or PTC124 for 48 h.

To determine the cellular expression of laminin β3 protein, cellular extracts were prepared 48 h after incubating with gentamicin as described and subjected to 4–15% SDS/PAGE (Bio-Rad). Proteins were then electrotransferred onto a nitrocellulose membrane. The presence of laminin β3 monomer was detected with monoclonal anti-laminin β3 antibodies (Anti-Kalinin B1, clone 17; BD Biosciences). The presence of laminin 332 trimer was detected with either polyclonal anti-laminin 332 (β) chain antibodies (Anti-Laminin β3/Laminin 5; Origene) or monoclonal anti-laminin 332 (γ) chain antibodies [Anti-Laminin-5 (g2 chain), clone D4B5; EMD Millipore] followed by secondary antibodies using either horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG and an enhanced chemiluminescence detection reagent (GE Healthcare).

To determine whether gentamicin-induced laminin 332 is secreted into the medium, the cells were treated with gentamicin for 48 h. The media were then collected and concentrated 10- to 15-fold using Amicon Ultra-4 Centrifugal Filters (Merck Millipore). Samples were then subjected to 4–12% SDS/PAGE followed by immunoblot analysis, as described above.

To evaluate the effects of gentamicin administration frequency on H-JEB1 and H-JEB2 cells, growth media were supplemented for the standard 48 h used in previous experiments. After 48 h, the media were supplemented daily with gentamicin for up to three additional days as indicated. Respective lysates and media were collected 24 h after their last indicated gentamicin treatment.

The sustainability of a single course of gentamicin on H-JEB1 and H-JEB2 cells was evaluated after treatment with gentamicin for the standard 48 h used previously. Media were then changed to fresh media without gentamicin for the duration of the experiment. Growth medium was replaced every other day until the conclusion of the experiment.

Cellular extracts were prepared and conditioned media were collected and concentrated as described above for evaluation at various time points for laminin β3 expression. These samples were subjected to 4–12% SDS/PAGE followed by immunoblot analysis, as previously described (26).

Cell Morphology and Proliferation Assay. We studied cellular morphology using a Leica DMIL LED inverted microscope (10× lens) equipped with a Leica DFC340 FX digital camera to digitally capture the images.

To assess the effects of gentamicin on H-JEB cell proliferation, H-JEB keratinocytes with stably integrated cDNA expression constructs containing nonsense mutations or normal human keratinocytes were seeded to uncoated, 24-well plates in quadruplicate at a density of 75,000 keratinocytes per well. Twenty-four hours after seeding, the media were changed to media containing gentamicin (0–1 mg/mL) where appropriate. At 48 h, cells were collected by trypsinization and incubated with 0.2% trypan blue, and live cells were manually counted.

Cell Viability. To evaluate gentamicin cytotoxicity, H-JEB1 and H-JEB2 keratinocytes were seeded to an uncoated, 96-well plate at a density of 1×10^4 cells per well in 100 μL of culture medium. Twenty-four hours later, media were changed and supplemented with gentamicin at indicated concentrations from 0 to 2 mg/mL or with amlexanox from 0 to 60 μg/mL (0–200 μM). Plates were allowed to incubate for 48 h. A freshly prepared solution of 4 mg 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-5[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT; VWR) in 4 mL of culture medium was mixed with 10 μL of phenazine methosulfate (PMS; Sigma) solution (3 mg of PMS in 1 mL of PBS), and 25 μL of the combined XTT/PMS solution was directly added to each 100-μL cell culture (51). Cultures were incubated for 4 h at 37 °C, and absorbance was read at 450 nm.

Cell Detachment. To determine the degree of gentamicin-induced cellular adherence, a trypsin-based detachment assay was employed (52). Briefly, normal human keratinocytes and H-JEB, H-JEB1, and H-JEB2 keratinocytes were seeded on 24-well tissue culture plates at a density of 1×10^4 cells per well. Twenty-four hours after seeding, the medium was changed to one containing gentamicin (500 μg/mL) where appropriate. After 72 h, 250 μL of trypsin/EDTA was added to each well, and any detached cells were removed after 5 min and counted. An additional 250 μL of trypsin/EDTA was added, and all remaining cells were allowed to detach and then counted. The percentages of cells detached were obtained, and the averages and SDs from nine independent wells for each condition per cell line were calculated.

Cell Migration. Cell migration was assessed as previously described (53). Briefly, colloidal gold salts were immobilized on coverslips and coated with type I collagen (15 μg/mL). Normal human keratinocytes, untreated parental H-JEB keratinocytes, or gentamicin-treated H-JEB1/H-JEB2 keratinocytes were suspended, plated on the coverslips, and allowed to migrate for 16–20 h. The cells were fixed in 0.1% formaldehyde in PBS and examined under dark-field optics with a video camera attached to a computer equipped with image capture capability. The computer analyzes 15 nonoverlapping fields in each experimental condition with NIH Image 1.6 and determines the percentage area of each field consumed by cell migration tracks (MI). Confirmation of a difference in migration as statistically significant requires rejection of the null hypothesis of no difference between mean MIs obtained from replicate sets at the $P = 0.05$ level with a Student's t test.

Immunofluorescence Staining of Cell Cultures. Normal human keratinocytes and H-JEB1/H-JEB2 keratinocytes previously treated (500 μg/mL gentamicin) or untreated were plated in TissueTek chamber slides (Nunc, Inc.) on polylysine at 37 °C for 18 h. Cells were immersed in periodate-lysine-paraformaldehyde fixative for 10 min at room temperature, washed several times with PBS to remove fixative, and then permeabilized and blocked by incubating in PBS with 3% BSA, 1% saponin, and 10% normal goat serum for 15 min at room temperature. The cells were incubated with one of two affinity-purified antibodies: monoclonal anti-laminin β3 antibodies at a 1:1,000 dilution or polyclonal anti-laminin 332(β) chain antibodies at a 1:800 dilution. Incubation occurred in a humidified chamber for 2 h, and cells were then washed three times with PBS and 1% saponin; they were subsequently counterstained with an FITC-conjugated goat antibody to mouse IgG (1:1,000; Organon Teknika-Capel) or CY3-conjugated goat antibody to rabbit (1:1,000; Organon Teknika-Capel) for 1 h and then washed. The cells were then examined and photographed with a Zeiss epiluminating immunofluorescence microscope. All images were photographed using the same camera and at identical exposure times.

Establishment of in Vitro Organotypic SEs and Immunofluorescence Microscopy. Establishment of an in vitro skin coculture model was performed as previously described (31). Briefly, DMEM medium (Corning, Discovery Labware) containing human dermal fibroblasts (1×10^6 cells per milliliter) was mixed with rat tail collagen 1 solution (2.5 mg of collagen per milliliter; Corning, Discovery Labware) and 10× DMEM (Corning, Discovery Labware). This solution was neutralized with sodium bicarbonate, 1 mL of this solution was distributed into each 12-well insert (ThinCerts 12-well, 3 μM pore size; Greiner Bio-One), and the gels were allowed to polymerize. The fibroblast-infused collagen gel was then submerged in serum-free DMEM and incubated for 24 h. The DMEM medium was then removed, and each dermal equivalent was covered with 50 μL of 50 μg/mL fibronectin (Sigma) in ultrapure water solution and allowed to incubate for 30 min. In the meantime, IKCs, H-JEB1 (pretreated with gentamicin or untreated), and H-JEB2 (pretreated with gentamicin or untreated) were resuspended in Epilife media supplemented with HKGS + 5% FCS (Thermo Fisher Scientific) at a concentration of 1×10^6 cells per milliliter. Cells in this solution were seeded onto each gel and incubated for 45 min to allow cells to adhere. Afterward, the gels were submerged in Epilife media supplemented with HKGS + 5% FCS and cultured for up to 2 wk with descending FCS concentrations (5%, 2%, and 0% FCS) every 2–3 d when media changes occurred, with gentamicin supplementation where appropriate. Between days 7 and 14 after keratinocyte seeding, SEs were extracted and placed on nitrocellulose strips after being soaked in PBS. SEs harvested within this time frame typically display an epidermis with a thickness of one to two keratinocytes (31). The SEs were soaked in 50% sucrose solution for 90 min and then slow-frozen on a metal plate over dry ice. Frozen SEs were mounted in optimum cutting temperature compound (OCT) and frozen. Five-micrometer-thick sections from the OCT-embedded SEs were cut using a cryostat, fixed for 5 min in cold acetone, and air-dried. Immunolabeling of SEs was performed using standard immunofluorescence methods, as described previously (54). SE sections were labeled with either polyclonal anti-laminin 332(β) chain antibodies followed by a CY3-conjugated goat anti-rabbit IgG (1:1,000) or colabeled with monoclonal anti-laminin 332(α) chain antibodies (anti-hLaminin-α3, clone P3H9-2; R&D Systems) and either polyclonal anti-Integrin β4 (Santa Cruz Biotechnology) or monoclonal rat anti-Integrin α6 (Santa Cruz Biotechnology) antibodies followed by FITC-conjugated goat antibody to mouse IgG (1:1,000 dilution) and CY3-conjugated goat-anti rabbit (1:1,000 dilution) or CY3-conjugated goat-anti rat (1:1,000 dilution), respectively. Representative photographs from stained sections were taken using a Zeiss Axioplan fluorescence microscope equipped with a Zeiss Axiocam MRM digital camera system. All images were photographed using the same camera and at identical exposure times. Mean fluorescence intensity at the DEJ was calculated for each sample using ImageJ (NIH; https://imagej.nih.gov/ij/), as described previously (19, 26).

ACKNOWLEDGMENTS. This work was supported, in part, by grants from Epidermolysis Bullosa Research Partnership and Epidermolysis Bullosa Medical Research Foundation (to M.C. and D.T.W.) and a Veterans Affairs Merit Award (to D.T.W.).

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