

Nonsense Suppressor Therapies Rescue Peroxisome Lipid Metabolism and Assembly in Cells From Patients With Specific *PEX* Gene Mutations

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ABSTRACT

Peroxisome biogenesis disorders (PBDs) are multisystemic autosomal recessive disorders resulting from mutations in *PEX* genes required for normal peroxisome assembly and metabolic activities. Here, we evaluated the potential effectiveness of aminoglycoside G418 (geneticin) and PTC124 (ataluren) nonsense suppression therapies for the treatment of PBD patients with disease-causing nonsense mutations. PBD patient skin fibroblasts producing stable *PEX2* or *PEX12* nonsense transcripts and Chinese hamster ovary (CHO) cells with a *Pex2* nonsense allele all showed dramatic improvements in peroxisomal very long chain fatty acid catabolism and plasmalogen biosynthesis in response to G418 treatments. Cell imaging assays provided complementary confirmatory evidence of improved peroxisome assembly in G418-treated patient fibroblasts. In contrast, we observed no appreciable rescue of peroxisome lipid metabolism or assembly for any patient fibroblast or CHO cell culture treated with various doses of PTC124. Additionally, PTC124 did not show measurable nonsense suppression in immunoblot assays that directly evaluated the read-through of *PEX7* nonsense alleles found in PBD patients with rhizomelic chondrodysplasia punctata type 1 (RCDP1). Overall, our results support the continued development of safe and effective nonsense suppressor therapies that could benefit a significant subset of individuals with PBDs. Furthermore, we suggest that the described cell culture assay systems could be useful for evaluating and screening for novel nonsense suppressor therapies. *J. Cell. Biochem.* 112: 1250–1258, 2011. © 2010 Wiley-Liss, Inc.

KEY WORDS: PEROXISOME BIOGENESIS DISORDER; ATALUREN; GENETICIN; NONSENSE MUTATION

Aminoglycoside antibiotics, such as gentamicin and its analog G418 (geneticin), can promote the translational read-through of stop codons (i.e., nonsense suppression) in cultured human cells and genetically engineered mouse models, as reviewed in Zingman et al. [2007] and Hainrichson et al. [2008]. This has led to small-scale clinical studies to evaluate gentamicin treatments in patients with nonsense mutations that result in cystic fibrosis [Wilschanski et al., 2000, 2003; Clancy et al., 2001; Linde et al., 2007; Sermet-Gaudelus et al., 2007], Duchenne and Becker muscular dystrophy [Wagner et al., 2001; Politano et al., 2003; Malik et al., 2010], hemophilia A and B [James et al., 2005], factor VII deficiency [Pinotti et al., 2006], Hailey-Hailey disease [Kellermayer et al., 2006], and McArdle disease [Schroers et al.,

2006]. To date, no lasting clinical benefits have been reported for individuals receiving gentamicin nonsense suppression therapies. Furthermore, their long-term toxicity and route of administration impede their clinical applications for chronic genetic disorders [Martinez-Salgado et al., 2007; Guthrie, 2008].

PTC124 (ataluren) is an orally delivered non-aminoglycoside compound reported to promote the translational read-through of premature stop codons [Welch et al., 2007]. As a result of its observed activity in primary human cells and mouse models, PTC124 is currently in clinical trials for the treatment of cystic fibrosis and Duchene's muscular dystrophy caused by nonsense mutations [Hirawat et al., 2007; Kerem et al., 2008]. PTC124 has demonstrated favorably low toxicity in these trials [Hirawat

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et al., 2007; Kerem et al., 2008]. Similar to observations made for gentamicin [Clancy et al., 2001; Wilschanski et al., 2003; Sermet-Gaudelus et al., 2007], CF patients receiving PTC124 have shown reduced electrophysiological abnormalities and increased apical CFTR protein expression in their nasal epithelial tissue [Kerem et al., 2008; Sermet-Gaudelus et al., 2010]. However, the clinical efficacy of PTC124 for the treatment of CF or DMD has not yet been reported in the scientific literature.

Peroxisomal biogenesis disorders (PBDs) are a group of autosomal recessive multisystemic disorders that are promising targets for nonsense suppressor therapies [Weller et al., 2003; Wanders, 2004; Steinberg et al., 2006]. Approximately 80% of PBD cases fall within the Zellweger spectrum (PBD-ZSD) category with the remaining cases classified as rhizomelic chondrodysplasia punctata type 1 (RCDP1) [Steinberg et al., 2004]. Over 90% of PBD-ZSD patients have mutations in a group of five *PEX* genes (*PEX1*, *PEX6*, *PEX10*, *PEX12*, and *PEX26*) crucial for normal peroxisome assembly and function [Steinberg et al., 2004]. Based on data from recent studies [Steinberg et al., 2004; Yik et al., 2009], nonsense mutations account for ~10% of the disease-causing *PEX* alleles found in PBD-ZSD patients. RCDP1 is an especially attractive candidate for nonsense suppressor therapies since the common *PEX7* L292X nonsense mutation, accounting for over 60% of mutant alleles [Braverman et al., 2000, 2002; Motley et al., 2002], yields a stable transcript [Braverman et al., 2002]. Since PBD patients with residual mutant *PEX* gene function tend to have milder medical conditions [Moser, 1999; Steinberg et al., 2006], a modest rescue of *PEX* gene activity could provide considerable clinical benefits for patients with more severe forms of disease.

Here, we used complementary experimental approaches to explore the potential effectiveness of nonsense suppressor therapies for PBDs. We evaluated peroxisome lipid metabolism and assembly in a collection of PBD patient skin fibroblasts with *PEX* gene nonsense mutations before and after G418 and PTC124 treatments. These functional assays focus on the downstream biochemical and cellular consequences of *PEX* gene mutations. In parallel, we used immunoblot assays to evaluate directly the activity of PTC124 in promoting the translational read-through of *PEX7* nonsense mutations found in RCDP1 patients. Collectively, our studies provide evidence that the development of safe and effective nonsense suppressor therapies could benefit significant numbers of PBD patients.

MATERIALS AND METHODS

CELL CULTURE AND DRUG TESTING

Primary fibroblast cultures from patients and healthy controls were obtained from the Peroxisomal Disease Laboratory at the Kennedy Krieger Institute and Coriell Institute Cell Repository, respectively. Prior to testing, cells were grown to confluence for 48 h at 37°C with 5% CO₂, as described [Karaman et al., 2003]. G418 was obtained from Sigma-Aldrich and PTC124 from Enzo Life Sciences and Dr. Christopher Austin at the National Institutes of Health. The 10 mg/ml G418 and 10 mM PTC124 stock solutions were freshly made in 1× PBS and DMSO, respectively.

QUANTITATIVE REAL-TIME PCR

Fibroblasts from patients and healthy controls were grown to confluence for 48 h and total RNA was extracted by the RNA STAT-60™ (Tel-Test) method. cDNA was prepared with the iScript™ cDNA Synthesis Kit (Bio-Rad) and PCR reactions were performed in triplicate with SYBR Green PCR master mix and reverse transcriptase (Applied Biosystems) with *PEX* gene specific primers. RT-PCR reactions were run on a DNA Engine Opticon 2 system (MJ Research).

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Fibroblasts and CHO cells were plated on 6-well plates for 24 h at 37°C and treated in duplicate with either 10 or 50 µg/ml of G418 or PBS media for 3 or 10 days. Fibroblasts were also seeded as above and treated in duplicate with 50 µM PTC124 or DMSO media for 3 or 10 days. Cells were processed and data collected as previously reported [Yik et al., 2009].

FATTY ACID AND PLASMALOGEN ANALYSES

Drug-containing media were replenished every 3 days until cells were assayed at the designated 3 or 10-day time point. Cell extracts were processed and total fatty acids analyzed using capillary GC with flame ionization detection, as described [Moser and Moser, 1991]. C16:0 DMA and C18:0 DMA levels also were measured by capillary GC with flame ionization detection, as described [Bjorkhem et al., 1986; Steinberg et al., 2008].

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PEX7 mutant cDNAs were constructed by site-directed mutagenesis using wild-type *PEX7* as template. They were inserted N-terminal to and in-frame with Gal4 BD present in the pBIND plasmid (Promega) to create different pBIND-*PEX7* constructs. Cultured HEK293 cells were transfected in triplicate using 0.25 µg of jetPEI (Polyplus-Transfection, Inc.) with the designated pBIND-*PEX7* construct. Twenty-four hours post-transfection, cells were treated with PTC124 (10, 50 µM). Whole cell lysates were obtained for immunoblotting using anti-Gal4 antiserum (Millipore) to detect the designated BIND-*PEX7* protein. Experiments were performed in triplicate and repeated four times.

RESULTS

We chose cultured skin fibroblasts from four healthy controls, seven PBD-ZSD patients with two previously confirmed mutant alleles in the *PEX2*, *PEX10*, *PEX12*, or *PEX26* genes, and one RCDP1 patient that was homozygous for the common *PEX7* L292X mutation [Moser, 1999; Steinberg et al., 2006] (Table I, cultures A–H). This includes cultures from six PBD-ZSD patients with at least one *PEX* gene nonsense mutation and a patient with two *PEX2* frame-shift mutations (culture D), which served as a negative control. In addition, we selected the *Per2* (R123X) null Chinese hamster ovary (CHO) ZR-82 cell line (Table I), previously reported to show improved peroxisomal lipid metabolic functions in response to G418 treatment and the wild-type CHO K1 parental line [Allen and Raetz, 1992].

TABLE I. PBD-ZSD and RCDP1 Patient Skin Fibroblast and Mutant CHO Cell Cultures Evaluated

Culture	Mutant gene ^a	Genotype allele 1			Genotype allele 2			Level ^c
		Coding	Context ^b	Protein	Coding	Context ^b	Protein	
A ^d	<i>PEX2</i>	355C>T	GAA CGA TGA	R119X	355C>T	GAA CGA TGA	R119X	87%
B ^d	<i>PEX2</i>	355C>T	GAA <u>CGA</u> TGA	R119X	355C>T	GAA <u>CGA</u> TGA	R119X	77%
C	<i>PEX2</i>	373C>T	UUU <u>CGA</u> AAC	R125X	373C>T	UUU <u>CGA</u> AAC	R125X	103%
D	<i>PEX2</i>	273delT	—	P91fs	273delT	—	P91fs	54%
E	<i>PEX7</i>	875T>A	GGU UUA GAC	L292X	875T>A	GGU UUA GAC	L292X	50%
F	<i>PEX10</i>	4delG	—	A2fs	892G>T	CTG <u>GAG</u> GAG	E298X	23%
G	<i>PEX12</i>	538C>T	CUU CGA UAC	R180X	887_888delTC	—	L296fs	48%
H	<i>PEX26</i>	296G>A	CGG <u>UGG</u> CAA	W99X	296G>A	CGG UGG CAA	W99X	25%
ZR-82 ^e	<i>Pex2</i>	367C>T	TTT <u>CGA</u> AAC	R123X		Presumed deleted null allele		NT

NT, not tested.

^aRefSeq IDs: *PEX2* (NM_000318.2), *PEX10* (NM_153818.1), *PEX12* (NM_000286.2), and *PEX26* (NM_017929.4).

^bCodons (5'–3') affected by nonsense mutations (mutated base underlined) and flanking codons are provided. "—" = frame-shift mutation.

^cAverage percent abundance of mutated PEX transcripts relative to the cognate PEX transcripts in four healthy controls. All quantitative PCR experiments were performed in triplicate.

^dDerived from different PBD-ZSD patients with the same *PEX2* mutations.

^eChinese hamster ovary (CHO) cells.

PEX GENE EXPRESSION IN CULTURED FIBROBLASTS

Based on global gene expression profiling data, cultured fibroblasts from healthy donors show abundant *PEX2* transcript levels (~90th percentile of all transcripts surveyed), but more modest levels of the *PEX7*, *PEX10*, *PEX12*, or *PEX26* transcripts (30–70th percentile) (Supplemental Table I). Using quantitative PCR assays, we found that fibroblasts that were homozygous for *PEX2* or *PEX7* nonsense mutations (cultures A, B, C, and E), homozygous for *PEX2* frame-shift mutations (culture D), or compound heterozygous for *PEX12* nonsense and frame-shift alleles (culture G) produced substantial levels of mutant *PEX* gene transcripts (i.e., at least 40% of those in healthy controls) (Table I). This finding is consistent with the prediction that these mutant transcripts would not undergo nonsense-mediated decay (NMD) due to their sequence context [Bhuvanagiri et al., 2010]. We prioritized cultures A, B, C, and E for drug testing since their homozygous, stable nonsense *PEX* transcripts should provide the greatest opportunity for producing biologically significant levels of the corresponding full-length PEX protein. We also prioritized culture G since it is highly likely that the *PEX12* nonsense allele is producing stable mutant transcript. Finally, we note that culture D serves as a rigorous negative control since its frame-shift alleles produce stable transcript.

CELL VIABILITY IN RESPONSE TO DRUG TREATMENTS

We used MTT assays [Mosmann, 1983] to evaluate the viability of cultured fibroblasts from patients (A–H) and three healthy controls after drug treatments (Supplemental Fig. 1). All cultures treated with low dose G418 (5 and 10 μg/ml) showed over 70% viability at 3 days and over 40% viability at 10 days. Cultures treated with high dose G418 (50 μg/ml) showed non-uniform viability at 3 days and consistently low viability at 10 days (15% average viability). All cultures exposed to low dose PTC124 (50 μM) showed over 80% viability at 3 and 10 days. In exploratory studies, the 100 μM PTC124 dose lowered cell viability at 3 and 10 days (58% and 35% average viability, respectively). High dose PTC124 (250 μM) was toxic to all cultures tested at both times (less than 10% viability at 10 days). Finally, CHO ZR-82 and K1 cells treated with 5, 10, or 50 μg/ml G418 or 50 μM PTC124 showed over 70% viability at 3 and 10

days. Collectively, the results from these MTT assays were used to inform the doses chosen for this study and aid in the interpretation of responses to drug treatments.

RESCUE OF VLCFA CATABOLISM

Consistent with the known defects in peroxisomal β-oxidation of very long chain fatty acids (VLCFA) [Steinberg et al., 2006], cultured fibroblasts from PBD-ZSD patients and CHO ZR-82 cells had over threefold elevated C26:0/C22:0 fatty acid ratios relative to healthy controls. As expected, culture E from a RCDP1 patient had C26:0/C22:0 fatty acid ratios similar to those of healthy controls. This reflects the fact that RCDP1 patients have normal VLCFA β-oxidation. In singleton exploratory experiments evaluating cultures A–H and CHO ZR-82 cells treated with 50 μg/ml G418 for three days, PBD-ZSD cultures A and C with *PEX2* nonsense mutations and PBD-ZSD culture G with *PEX12* nonsense mutations showed ≥25% decreases in their C26:0/C22:0 ratios (Supplemental Table II). In similar exploratory studies, none of the PBD cell cultures (A–H) or CHO-ZR-82 cells treated with 50 μM PTC124 for 3 days showed this type of response (Supplemental Table II).

To follow-up on cultures showing the most promising rescue of VLCFA catabolism, we tested in triplicate the effects of 3- and 10-day G418 and PTC124 treatments on the *PEX2* (culture A), *PEX2* (culture C), and *PEX12* (culture G) mutant fibroblasts (Table II). We also included CHO ZR-82 cells due to literature precedent [Allen and Raetz, 1992]. Prior to drug treatment, all patient cultures showed over sixfold elevated C26:0/C22:0 ratios relative to the healthy control. Following 10-day G418 treatment (5 and 10 μg/ml), all patient cultures demonstrated substantial decreases in their C26:0/C22:0 ratios (Fig. 1). Cultures A and G now had (i) less than 3.5-fold elevated C26:0/C22:0 ratios relative to the drug-treated healthy control and (ii) at least 53% decreases in their C26:0/C22:0 ratio compared to PBS vehicle control ($P < 0.001$). However, culture D with two *PEX2* frame-shift mutations and healthy control cultures showed no significant response to 10-day G418 treatments (5 and 10 μg/ml) (Table II). Untreated CHO ZR-82 cells had a 25.5-fold elevated C26:0/C22:0 ratio relative to CHO K1 wild-type cells. After 10-day treatments with 50 μg/ml G418, CHO ZR-82 cells had (i) only

TABLE II. Summary of Biochemical Rescue in PBD Patient Fibroblast Cultures

Culture	Treatment	Evidence of biochemical rescue ^a					
		C16:0 DMA, 3 days ^b	C16:0 DMA, 10 days ^b	C18:0 DMA, 3 days ^b	C18:0 DMA, 10 days ^b	VLCFA, 3 days ^c	VLCFA, 10 days ^c
A	5 µg/ml G418	--	+++	--	+++	--	+++
A	10 µg/ml G418	--	+++	+	+++	--	+++
A	100 µM PTC124	--	--	--	--	--	--
A	250 µM PTC124	--	+	--	--	--	--
C	5 µg/ml G418	--	+++	--	+++	--	++
C	10 µg/ml G418	--	+++	--	+++	--	++
C	100 µM PTC124	--	--	--	--	--	--
C	250 µM PTC124	--	--	--	--	--	--
D	5 µg/ml G418	--	--	--	--	--	--
D	10 µg/ml G418	--	--	--	--	--	--
E	5 µg/ml G418	--	--	--	--	NA	NA
E	10 µg/ml G418	--	--	--	--	NA	NA
E	100 µM PTC124	--	--	--	--	NA	NA
G	5 µg/ml G418	--	+	--	++	--	++
G	10 µg/ml G418	--	+	--	++	--	+++
G	100 µM PTC124	--	--	--	--	--	--
G	250 µM PTC124	--	--	--	--	--	--

^aAll samples were treated with drug or vehicle controls for 3 and 10 days in triplicate.

^bDesignated DMA levels are >75% (+++), 50–75% (++), 25–50% (+), or <25% (–) of drug-treated healthy control with $P < 0.01$. “--” Indicates $P > 0.01$. P -values are calculated based on a two-tailed Student’s t -test.

^cC26:0/C22:0 ratio is 2- to 3-fold (+++), 3- to 4-fold (++), 4- to 5-fold (+), or >5-fold (–) elevated relative to drug-treated healthy control with $P < 0.01$. “--” Indicates $P > 0.01$. “NA” indicates not applicable as *PEX7* mutants are expected to show similar VLCFA ratios relative to healthy controls.

a 17.8-fold elevated C26:0/C22:0 ratio relative to drug-treated CHO K1 cells and (ii) a 78% decrease in C26:0/C22:0 ratio compared to PBS vehicle control ($P < 0.001$). In contrast, PTC124 treatments (100 and 250 µM) did not result in significantly decreased C26:0/C22:0 ratios (over 50% reduction, $P < 0.01$) for any human or CHO cell tested at 3 and 10 days.

RESCUE OF PLASMALOGEN BIOSYNTHESIS

Plasmalogens are ether glycerophospholipids whose synthesis is dependent upon peroxisome activity [van den Bosch et al., 1992; Gorgas et al., 2006; Braverman et al., 2010]. We evaluated

plasmalogen abundance in cultured cells by measuring amounts of C16:0 and C18:0 fatty alcohols attached to the sn-1 position of the glycerol backbone. These are detected as dimethyl acetal (DMA) derivatives after acid methanolysis of the vinyl-ether bond. In agreement with prior reports [Steinberg et al., 2006], PBD-ZSD and RCDP1 fibroblasts had over 45% reductions in C16:0 DMA and C18:0 DMA levels relative to healthy controls. In singleton exploratory experiments, control fibroblasts or wild-type CHO cells showed less than 20% increases in their C16:0 DMA and C18:0 DMA levels after 3-day treatments with 50 µg/ml G418 or 50 µM PTC124 (Supplemental Table II). However, patient cells with *PEX2*

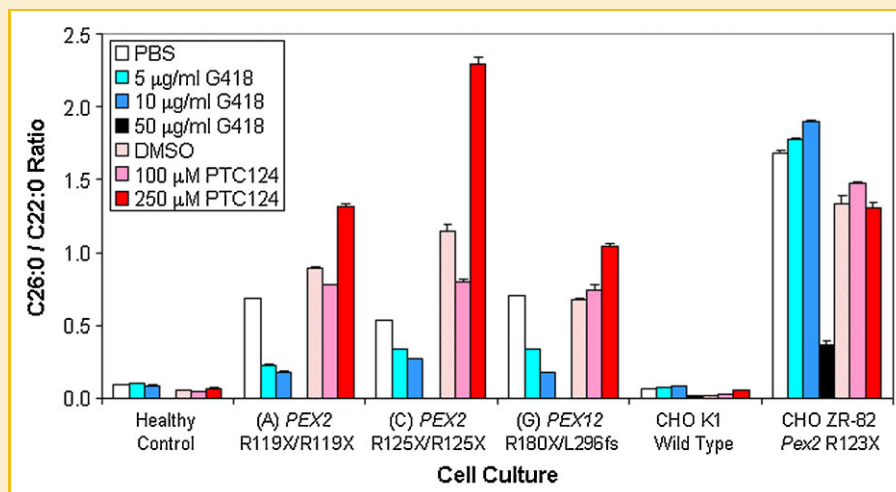


Fig. 1. Rescue of VLCFA metabolic activity in PBD patient fibroblasts and CHO ZR-82 cells. We report the average C26:0/C22:0 fatty acid ratios (y-axis) for healthy control fibroblast cultures, *PEX* mutant fibroblast cultures, CHO K1 wild-type cells, and CHO ZR-82 *Pex2* mutant cells treated with G418, PTC124, or appropriate vehicle control (0.1% PBS or 2.5% DMSO) for 10 days. The culture designation (Table I) and genotype of the treated cells are provided on the x-axis. All experiments were conducted in triplicate and error bars represent the standard error of the mean (SEM).

(cultures A, B, and C) and *PEX12* (culture G) nonsense mutations showed over 30% increases in C16:0 DMA and over 90% increases in C18:0 DMA levels after 3-day G418 treatments. While cultures A and B share the same *PEX2* nonsense mutations and both provide evidence of G418-mediated rescue of plasmalogen biosynthesis, we focused our follow-up analyses on culture A since it (unlike culture B) also showed a robust reduction in its C26:0/C22:0 ratio (Supplemental Table II). After 3-day treatments with 50 $\mu\text{g/ml}$ G418, CHO ZR-82 cells demonstrated an over 50% increase in C18:0 DMA levels, but no increase in C16:0 DMA levels (Supplemental Table II). Only PBD-ZSD culture A and RCDP1 culture E provided promising preliminary results in response to PTC124 treatments (47% and 28% increases in C16:0 DMA levels, and 53% and 24% increases in C18:0 DMA levels, respectively). However, culture A showed no evidence of increased VLCFA catabolism, as discussed above.

To rigorously evaluate plasmalogen biosynthesis rescue over extended periods of time, we measured in triplicate C16:0 DMA and C18:0 DMA levels in PBD-ZSD *PEX2* (cultures A and C) and *PEX12* (culture G) mutant cell cultures treated with 5 and 10 $\mu\text{g/ml}$ G418 or 100 and 250 μM PTC124 for 10 days (Fig. 2 and S2). All three G418-treated patient cells (A, C, and G) showed a robust rescue

of C16:0 DMA and C18:0 DMA levels (35–147% of drug-treated healthy controls). Additionally, all these cultures demonstrated significant increases in both C16:0 DMA and C18:0 DMA levels compared to PBS vehicle controls (2.5- to 6.8-fold and 3.8- to 8.2-fold, respectively, $P < 0.01$). In contrast, none of these cultures showed significant increases in C16:0 DMA and C18:0 DMA levels in response to PTC124.

In parallel, we treated *PEX7*-mutant RCDP1 patient fibroblasts (culture E) with 5 and 10 $\mu\text{g/ml}$ G418 or 100 μM PTC124 for 10 days (Supplemental Fig. 2). The RCDP1 patient fibroblasts treated with 10 $\mu\text{g/ml}$ G418 showed a modest increase (1.5-fold, $P < 0.01$) in C18:0 DMA levels relative to PBS vehicle controls. However, the C16:0 DMA and C18:0 DMA levels of these drug-treated RCDP1 patient fibroblasts were only 15–18% of the corresponding drug-treated healthy control fibroblasts. As above, these cells showed no significant increases in C16:0 DMA and C18:0 DMA levels in response to PTC124.

Consistent with prior reports [Allen and Raetz, 1992], CHO ZR-82 mutant cells treated with 50 $\mu\text{g/ml}$ G418 for 10 days showed dramatic rescue of C16:0 DMA and C18:0 DMA levels relative to drug-treated wild-type CHO K1 cells (82% and 57%, respectively) and significant improvement in C16:0 DMA and C18:0 DMA levels compared to PBS vehicle controls (5.6- and 3.3-fold increases respectively, $P < 0.001$) (Fig. 2). However, PTC124-treated CHO ZR-82 mutant cells showed no notable differences in C16:0 DMA or C18:0 DMA levels.

RESCUE OF PEROXISOME ASSEMBLY

We used immunofluorescence (IF) assays to evaluate peroxisome assembly in cultured cells. For PBD-ZSD patient cultures, which have defects in both the peroxisome targeting signal 1 (PTS1) and peroxisome targeting signal 2 (PTS2) pathways [Steinberg et al., 2006], we used PMP70 and catalase as markers for peroxisome membrane and matrix proteins, respectively. For RCDP1 cultures, which only have defects in the PTS2 import pathway [Steinberg et al., 2006], we used *PEX13* and thiolase as markers for peroxisome membrane and matrix proteins, respectively. In agreement with prior reports [Steinberg et al., 2006], PBD-ZSD and RCDP1 patient cells showed diffuse cytoplasmic localization of PTS1 and PTS2 matrix protein markers, respectively. For PBD-ZSD cells there were reduced numbers of normal peroxisomes, and an increased number of peroxisomal vesicles with no discernable evidence of matrix protein import. Three-day G418 (50 $\mu\text{g/ml}$) treatments of cultures A, C, E, F, and H did not result in a detectable improvement in peroxisome assembly (Supplemental Fig. 3). However, we found modest evidence of improved assembly for culture G in some experiments (Supplemental Fig. 4U), but not others (Supplemental Fig. 3J). PTC124 (50 μM) treatments did not result in an appreciable change in peroxisome assembly in any PBD-ZSD or RCDP1 fibroblast cultures tested (A, C, E, F, G, and H) (Supplemental Figs. 5, 6, and 8).

Given the positive results in the biochemical rescue of the *PEX2* (cultures A and C), and *PEX12* (culture G) mutant fibroblast cultures, we evaluated peroxisome assembly in cells treated with 10 and 50 $\mu\text{g/ml}$ G418 or 50 μM PTC124 for 3 and 10 days. We observed improved peroxisome assembly in the G418-treated *PEX2* mutant

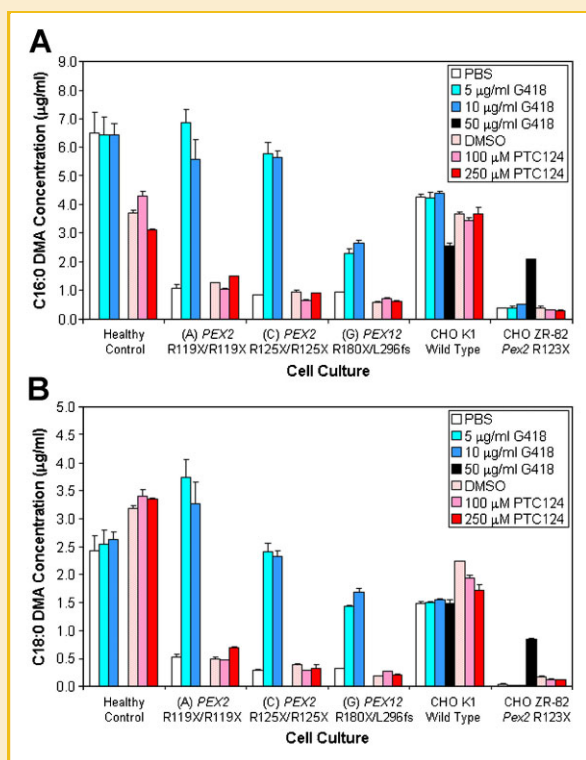


Fig. 2. Rescue of plasmalogen biosynthesis in PBD patient fibroblasts and CHO *Pex2* nonsense mutant cells. We report plasmalogen levels in healthy control fibroblast cultures, *PEX* mutant fibroblast cultures, CHO K1 wild-type cells, and CHO ZR-82 *Pex2* mutant cells treated with G418, PTC124, or appropriate vehicle control (0.1% PBS or 2.5% DMSO) for 10 days. For each culture, we provide the average (A) C16:0 DMA and (B) C18:0 DMA levels (y-axis). The culture designation (Table I) and genotype of the treated cells are provided on the x-axis. All experiments were conducted in triplicate and error bars represent the SEM.

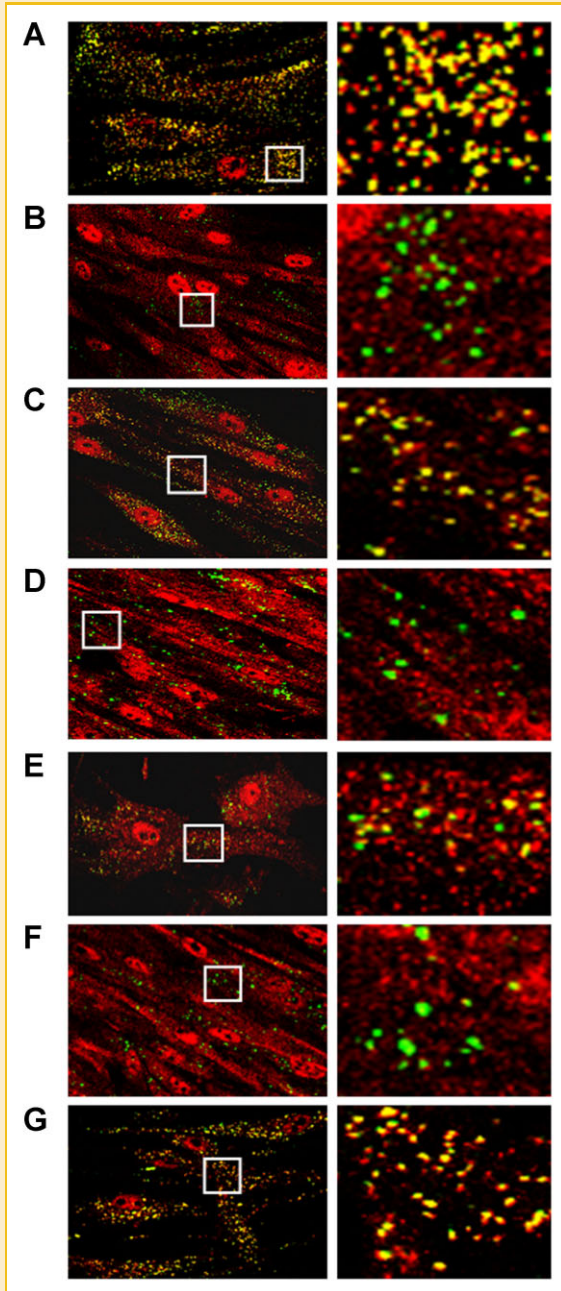


Fig. 3. Peroxisome assembly in G418-treated PBD patient fibroblasts. We conducted immunofluorescence-based peroxisome assembly assays on patient fibroblasts treated with indicated vehicle control or G418 for ten days. Anti-human PMP70 (green) and catalase (red) antibodies were used to highlight the peroxisome membrane and matrix proteins, respectively. Results from (A) healthy control fibroblasts treated with PBS vehicle control, (B) *PEX2* p.R125X/p.R125X culture C treated with PBS vehicle control, (C) culture C treated with 50 μ g/ml G418, (D) *PEX2* p.R119X/p.R119X culture A treated with PBS vehicle control, (E) culture A treated with 50 μ g/ml G418, (F) *PEX12* p.R180X/p.L296fs culture G treated with PBS vehicle control, (G) culture G treated with 50 μ g/ml G418 are provided. Punctate yellow staining indicates co-localization of peroxisome membrane and matrix markers. Enlarged views of the white boxed areas in the left panels are provided in the adjacent right panels. The vehicle control is 0.5% PBS.

cultures A and C at 10 days (Fig. 3C,E, and Supplemental Figs. 4K,L,Q,R) and *PEX12* (culture G) mutant fibroblasts at both 3 and 10 days (Fig. 3G and Supplemental Fig. 4U,W,X). PBD-ZSD cultures C and G were almost indistinguishable from control cultures after 10-day 50 μ g/ml G418 treatment (Fig. 3C,G). Marginal improvements were observed for culture A in response to 10-day G418 treatments. In contrast, cultures A, C, and G treated with PTC124 showed no discernable improvements in peroxisome assembly (Supplemental Fig. 6).

Additionally, neither G418 nor PTC124 treatment rescued peroxisome assembly in RCDP1 patient fibroblasts at 3 or 10 days (Supplemental Figs. 7 and 8). Finally, in agreement with prior studies [Allen and Raetz, 1992], we observed no rescue of peroxisome assembly in G418-treated *Pex2*-R123X CHO cells, despite the observed biochemical rescue (Supplemental Fig. 9).

PEX7 REPORTER GENE MODEL SYSTEM

Given that over 60% of RCDP1 patients could benefit from non-toxic nonsense suppressor therapies [Braverman et al., 2000, 2002; Motley et al., 2002], we developed reporter gene assays to reevaluate if PTC124 could efficiently promote the translational read-through of three naturally occurring *PEX7* nonsense alleles, including the common L292X mutation (Fig. 4). HEK293 cells were transfected with a pBIND-*PEX7* plasmid that expresses a Gal4-*PEX7* fusion protein containing a *PEX7* nonsense mutation and then cultured in PTC124 (10 or 50 μ M) for 72 h. Afterwards, immunoblotting was performed on whole cell lysates with anti-Gal4 antibody. Although all truncated forms of the *PEX7*-mutant proteins were present, there was no evidence of full-length Gal4-*PEX7* fusion protein production in the drug-treated cells.

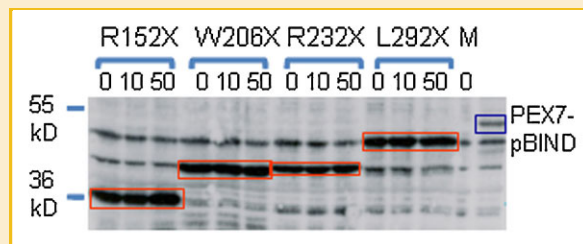


Fig. 4. Read-through of *PEX7* nonsense alleles in PTC124-treated cells. HEK293 cells were transfected with *PEX7*-pBIND constructs that express Gal4-*PEX7* fusion proteins of the indicated *PEX7* p.R152X, p.W206X, p.R232X, and p.L292X genotypes (see the Materials and Methods Section for details). After 24 h, the transfected cell populations were cultured in 0, 10, or 50 μ M PTC124 for 72 h. Whole cell protein lysates (25 μ g per lane) were separated on agarose gels, transferred to a nylon membrane, and incubated with Gal4 antibody which detects all *PEX7*-pBIND fusion proteins. The *PEX7* genotype and drug concentration are provided on top of each lane. M = mock transfection. The final lane depicts results from cells transfected with a plasmid expressing full-length *PEX7*-pBIND (highlighted in blue box). Truncated mutant *PEX7*-pBIND proteins are highlighted in red boxes.

DISCUSSION

We demonstrated that cultured PBD-ZSD patient skin fibroblasts with stable *PEX2* or *PEX12* nonsense transcripts showed dramatic improvements in peroxisomal lipid catabolic and anabolic activities in response to the chosen G418 treatments, but not the PTC124 treatments investigated (Table II and Figs. 1 and 2). In all cases, G418-mediated rescue was observed by 10 days using treatment conditions that result in moderate reductions in cell viability (Supplemental Fig. 1). In follow-up cell imaging assays, we observed that the rescue of peroxisomal lipid metabolic functions was coupled with improved peroxisome assembly. This ranged from robust (culture G) to marginal (culture A) increases in vesicles that stain positive for peroxisome membrane (PMP70) and matrix (catalase) markers (Fig. 3 and Supplemental Fig. 4). These quantitative biochemical assays and qualitative peroxisome assembly assays provided complementary means of surveying peroxisome function and structure in drug-treated patient cells.

Given the unavailability of quality *PEX2* and *PEX12* antibodies for immunoblot or IF assays, we cannot formally state the mechanistic basis for the G418-mediated rescue of peroxisome lipid metabolic activities or assembly in patient cells. However, the nonsense suppressor activity of G418 has been demonstrated in multiple contexts, as reviewed in Zingman et al. [2007] and Hainrichson et al. [2008]. In addition, the decreased C26:0/C22:0 fatty acid ratio, increased C16:0 and C18:0 DMA levels, and improved peroxisome assembly in cultures A, C, and G provide compelling positive evidence of increased mutant *PEX* gene function. Furthermore, the non-responsiveness of culture D with two *PEX2* frame-shift mutations (culture D) to G418 treatments strongly suggests that the biochemical rescue observed in fibroblast cultures A and C with *PEX2* nonsense mutations is related to their genotypes. Likewise, healthy control fibroblasts show no substantive changes in peroxisomal lipid metabolism in response to G418, relative to PBS vehicle controls.

We included *Per2*-mutant CHO ZR-82 cells in our surveys due to a prior report that G418 treatment could rescue their peroxisomal lipid metabolic functions [Allen and Raetz, 1992]. In this initial publication, the genotype of these CHO ZR-82 cells and the potential mode of G418 activity were not discussed. Subsequently, the same group demonstrated that the CHO ZR-82 cells contained a *Per2* p.R123X nonsense mutation and that non-responsive *Per2*-mutant CHO cells in the original study had a *Per2* frame-shift mutation [Thieringer and Raetz, 1993]. Thus, we theorized that the G418-mediated biochemical rescue in CHO ZR-82 cells resulted from the nonsense suppressor activity of G418. In agreement with the prior study [Allen and Raetz, 1992], our G418-treated CHO ZR-82 cells showed a substantial rescue of VLCFA metabolism and plasmalogen biosynthesis. In contrast, the CHO ZR-82 cells were non-responsive to PTC124 treatments. In follow-up cell imaging assays, we found no discernable rescue of peroxisome assembly in G418-treated CHO ZR-82 cells, also in agreement with prior studies [Allen and Raetz, 1992]. This indicates that biochemical assays can be more sensitive than standard assembly assays for detecting low-level peroxisome functions.

In our synthetic model system, we could not detect PTC124 mediated read-through of *PEX7* nonsense mutations by immunoblotting. This strongly suggests that the lack of improved plasmalogen biosynthesis or peroxisome assembly in RCDP1 fibroblasts (culture E, homozygous for the common *PEX7* p.L292X mutation) in response to PTC124 treatments is not due to the production of non-functional full-length *PEX7* protein. Nevertheless, our immunoblotting assays may lack the sensitivity to detect small increases in full-length *PEX7* protein in response to PTC124 treatments.

The general non-responsiveness of PBD patient skin fibroblast and CHO ZR-82 cultures to PTC124 treatments could add to ongoing discussions concerning its mode of biological activity [Auld et al., 2009; Inglese et al., 2009; Peltz et al., 2009]. PTC124 was initially discovered based on its ability to promote firefly luciferase (FLuc) reporter gene activity in mammalian cell culture assays [Welch et al., 2007]. Although this increase was reportedly caused by the translational read-through of an engineered stop codon, PTC124 can directly increase the activity of the FLuc protein product under specific assay conditions [Auld et al., 2009]. Subsequently, it has been demonstrated that under certain circumstances, PTC124 can be converted to the acyl-AMP mixed anhydride adduct, PTC124-AMP, which can bind to and stabilize the FLuc protein and prevent other inhibitors from binding [Auld et al., 2010; Thorne et al., 2010]. This can result in increased FLuc activity in the absence of translational read-through. Nevertheless, PTC124 has been reported to promote the translational read-through in FLuc activity assays under different assay conditions [Peltz et al., 2009]. Furthermore, PTC124 has been reported to promote the read-through of premature stop codons in multiple other biological assay systems including DMD [Welch et al., 2007] and CF [Du et al., 2008] mouse models and myotubes from a patient with Miyoshi myopathy [Wang et al., 2010]. Most intriguingly, PTC124 treatments can result in substantially increased CFTR activity in a subset of CF patients with disease-causing nonsense mutations [Kerem et al., 2008; Sermet-Gaudelus et al., 2010].

The lack of evidence for PTC124 nonsense suppressor activity in our assays could be due to numerous factors, including the sequence context of the premature stop codons and treatment regimen. In one reporter gene model system (subject to interpretation as discussed above), PTC124 showed maximal activity to promote the read-through of a UGA stop codon with lesser activities for UAG and UAA stop codons [Welch et al., 2007]. Here, PBD-ZSD cultures A, B, C, G, and CHO ZR-82 cells had premature UGA stop codons (Table I), but did not show consistent increases in peroxisome metabolic functions in response to PTC124 treatments. In terms of PTC124 concentration, we chose the low exploratory (50 μ M) dose based on studies of primary human muscle cells [Welch et al., 2007] and the intermediate (100 μ M) and high (250 μ M) doses based on observed cell toxicity (Supplemental Fig. 1). Furthermore, we demonstrated that the 10-day time point was sufficient for the G418-mediated rescue of peroxisome metabolic functions and/or assembly in the discussed PBD-ZSD patient fibroblast cultures and CHO ZR-82 cells. While we cannot exclude the possibility that PTC124 has substantial nonsense suppressor activity in PBD patient cells under different treatment regimens, our results suggest

that any theoretical window of robust PTC124 nonsense suppressor activity in our cell culture models would be narrow or especially sensitive to assay conditions.

Based on the observed activity of G418 in our functional assays, we provide evidence that nonsense suppressor therapies could be beneficial to a subset of patients with PBDs. The development of safe and effective nonsense suppressor therapies for PBDs will continue to be challenging as numerous technical concerns needed to be addressed, including the ability to cross the blood-brain barrier. Regardless of the pharmacological properties of different nonsense suppressor therapies, it is likely that only patients with nonsense alleles yielding stable mutant transcripts could benefit from these interventions. Likewise, the identity of the amino acid incorporated at the premature stop codon would need to be compatible with protein function in order for any nonsense suppressor therapy to be effective. To help address these challenges, we have identified human and CHO cell culture model systems and functional assays that can be used to validate the effectiveness of candidate nonsense suppressor drugs. Other well-characterized assays of peroxisomal biochemical functions could add further value to these model systems [Gootjes et al., 2002; Wanders and Waterham, 2006; Watkins et al., 2010]. In the future, we believe cultured patient fibroblast and CHO cell model systems could be adapted for high-throughput drug screens to identify nonsense suppressor therapies and other drugs suitable for the treatment of peroxisomal disorders.

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