RESEARCH ARTICLE

A G542X cystic fibrosis mouse model for examining nonsense mutation directed therapies

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Abstract

Nonsense mutations are present in 10% of patients with CF, produce a premature termination codon in CFTR mRNA causing early termination of translation, and lead to lack of CFTR function. There are no currently available animal models which contain a nonsense mutation in the endogenous Cftr locus that can be utilized to test nonsense mutation therapies. In this study, we create a CF mouse model carrying the G542X nonsense mutation in Cftr using CRISPR/Cas9 gene editing. The G542X mouse model has reduced Cftr mRNA levels, demonstrates absence of CFTR function, and displays characteristic manifestations of CF mice such as reduced growth and intestinal obstruction. Importantly, CFTR restoration is observed in G542X intestinal organoids treated with G418, an aminoglycoside with translational readthrough capabilities. The G542X mouse model provides an invaluable resource for the identification of potential therapies of CF nonsense mutations as well as the assessment of in vivo effectiveness of these potential therapies targeting nonsense mutations.

Introduction

Cystic Fibrosis (CF) is an autosomal recessive genetic disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR is an anion channel expressed throughout the body with highest expression in epithelial tissues. Absence of CFTR function impairs transepithelial fluid and electrolyte permeation and results in viscous mucus along the epithelial lining of organs leading to a wide range of disease manifestations. Common manifestations of CF include lung failure [1–3], pancreatic insufficiency [4], intestinal obstruction [5–8], and reduced growth [9–11]. Over 2000 unique CFTR variants have been identified, with ~300 categorized as definitively CF-causing mutations [12, 13]. CF-causing nonsense mutations are the second most common CF mutation type, and are found in...
approximately 10% of patients with CF [14, 15]. Nonsense mutations create a premature termination codon (PTC) in CFTR, which leads to premature termination of CFTR translation [16].

Recently, there has been progress in the development of CFTR modulators that restore CFTR function in patients with specific CFTR mutations. Examples include VX-770 (ivacaftor) for gating mutations like G551D [17, 18] and VX-809 (lumacaftor) for the misfolding mutation F508del [19, 20]. However, no therapies are available to CF patients that restore function to nonsense mutations in CFTR. Certain drugs, like gentamicin and PTC124 (ataluren), have been shown to interact with the ribosome to induce readthrough of PTCs by allowing insertion of a near-cognate aminoacyl tRNA and translation of a full-length protein [21–23]. Both gentamicin and PTC124 have been used in clinical trials for patients with nonsense mutations [24–27]. However, gentamicin treatment is associated with nephrotoxicity and ototoxicity [28] and PTC124 has produced no significant improvements in adult CF patients with nonsense mutations in Phase 3 clinical trials [29]. Therefore, no currently available treatment for CF nonsense mutations is both safe and clinically effective.

Because of the safety issues surrounding PTC readthrough strategies, animal models of CFTR nonsense mutations are essential for in vivo validation of new therapies. Current CF mouse models with nonsense mutations were created in a way that either does not allow for correction of the nonsense mutation or [30, 31] or utilizes non-endogenous CFTR with tissue specific expression [14]. Therefore, a model with global expression of the G542X mutation in the endogenous mouse Cftr will have a greater value for examining the efficacy of nonsense mutation therapies on Cftr in multiple tissue types with native expression levels. In this manuscript, we describe the generation of a mouse model with expression of the G542X mutation, the most common nonsense mutation in CF [32], using CRISPR/Cas9 gene editing technology. Mice homozygous for the G542X mutation have reduced Cftr expression and absence of CFTR function in the airway and intestine. These mice display typical cystic fibrosis manifestations such as poor growth and reduced survival due to intestinal obstruction. Importantly, we demonstrate that pharmacological readthrough of the G542X nonsense mutation in this model allows production of functional CFTR. The G542X mouse will be a valuable model for the examination of nonsense mutation therapies for CF and other genetic diseases caused by nonsense mutations.

Materials and methods

Generation of the G542X allele

To produce the G542X mouse Cftr allele (Cftr\textsuperscript{G542X}) using the gene editing system CRISPR/Cas9, guide RNAs (gRNA) were selected in exon 12 of mouse Cftr using CRISPR design software that identifies optimal gRNAs based on proximity to target as well as off-target cutting predictions [33]. Four of these gRNAs were tested in vitro using guide-it gRNA in vitro transcription and screening kit (Clontech) for the ability to guide Cas9 nuclease activity to the desired DNA sequence. One gRNA (25 ng/ul; PNABio), a 120 bp single stranded oligonucleotide (ssODN) containing the G542X mutation (25 ng/ul; IDT) centered on the cut site and either Cas9 mRNA (25–50 ng/ul; PNABio) or Cas9 protein (25 ng/ul; PNABio) were injected in the pronucleus of C57BL/6 one-cell embryos. Embryos were then placed in pseudo-pregnant females to develop. Ear punches from the 22 resulting pups were sequenced using next generation sequencing. The region of interest was PCR amplified (~300 bp) with flanking primers that were tailed with a universal sequence. A secondary PCR amplification was then performed on the primary PCR product using a set of primers specific for the universal sequence and tailed with I5,I7 Illumina index and specific barcode sequences allowing for the
sequencing of multiple samples at the same time. The mixture of barcoded PCR products were analyzed on an Illumina MiSeq instrument using a paired-end 300 base pair kit allowing for 25 million reads per run. Data were de-convoluted and variant analysis in comparison to the original sequence was performed using the Outknocker software program [34].

Mice

In some experiments, the G542X mouse model was compared to a previously published CFTR null model carrying a S489X mutation (Cftr\textsuperscript{m1Unc}) which is congenic on the C57BL/6J background [31]. Mice homozygous for these mutations were created by breeding heterozygous males and females and wild type littersmates were used as controls. Genotyping was completed by PCR analysis using DNA extracts from ear biopsies. To detect the G542X allele (319 bp) primers P1 (5′ – ACAAGACAACACACGTTCTCT -3′) and P2 (5′ TCCATGCACCATAACAA CAAGT -3′) were used. To detect the wildtype (WT) allele (319 bp) P2 and P3 (5′ – ACAAGACAACAGTTCTTG -3′) were used in a separate reaction. PCR reactions were completed for 40 cycles of 95˚C for 30 seconds, 58˚C for 30 seconds and 72˚C for 30 seconds and products were run out on 2% agarose gels. All mice were allowed unrestricted access to water and solid chow (Harlan Teklad 7960; Harlan Teklad Global Diets). All animals were maintained on a 12-h light, 12-h dark schedule at a mean ambient temperature of 22˚C and were housed in standard polysulfone microisolator cages in ventilated units with corncob bedding. Animals were monitored on a daily basis, and weight was assessed every 5 days from 10 to 40 days of age. Length of 6-wk-old euthanized mice was assessed from nose to anus by use of digital calipers. For G418 treatment, mice were intraperitoneal injected on three consecutive days (25mg/kg b.w.). Twenty four hours after last injection, mice were sacrificed and lungs were flash frozen and RNA prepared as described below. The Institutional Animal Care and Use Committee of Case Western Reserve University approved all animal protocols.

Expression analysis

One μg of RNA was reversed transcribed in cDNA using QScript cDNA synthesis kit (VWR). Real-time quantitative PCR was performed on a StepOne PCR system (Applied Biosystems). Cftr expression was assessed using a TaqMan expression assay which used primers spanning exon 17 and 18 (Mm00445197; Applied Biosystems). Expression was normalized to β-actin as the endogenous control. Each RNA sample was used to make cDNA in duplicate and the expression results were then averaged to yield the final value. The average of each sample was then expressed as a percentage of wildtype expression.

Bioelectric measurements

Nasal potential difference (NPD) measurements were obtained as previously described [35, 36]. NPD (mV) was assessed after the addition of chloride-free HEPES-buffered saline containing 10 μM forskolin. Short circuit measurements on intestinal sections were obtained as previously described [36]. The change in short-circuit current was calculated after the addition of 10 μM forskolin and 100 μM IBMX to the basolateral side of the intestinal sections.

Intestinal organoid harvesting and culture

Intestinal organoids were harvested from adult mice as described by others [37]. Briefly, the mouse was sacrificed, the small intestine removed and flushed using PBS. The intestine was cut longitudinally, villi removed with a razor blade and the remaining intestine was cut into ~0.5 cm segments and suspended in Gentle Cell Dissociation Reagent (STEMCELL
Technologies) in a 50 mL conical tube for 30 minutes under gentle agitation with a shaker. In a sterile tissue culture hood, the intestinal segments were vigorously shaken by hand for 30 seconds, and the supernatant was deposited in a 10 cm dish. The segments were resuspended in ice-cold PBS lacking Mg** and Ca**, and vigorously shaken again for 30 seconds. This process was repeated until four fractions of supernatant were produced. The fraction which was most enriched for intestinal crypts was filtered using a 100 μm cell strainer, and pelleted at 450xg for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 500 μl of a 1:1 mixture of MatriGel (Corning) and Intesticult Organoid Growth Media (OGM; STEMCELL Technologies). Crypts were diluted to a concentration of 10 crypts/μl, and plated into non-tissue culture treated 24-well plates, with 35 μl of MatriGel:OGM mixture deposited in each well. The MatriGel:OGM was solidified at 37˚C for 15 minutes, then 500 μl of OGM was gently deposited in each well. The organoids were stored in a 37˚C incubator with 5% CO₂. The OGM was changed once every three days, and the organoids were passaged once every 7 days.

Measurement of intestinal organoid swelling

Forskolin-induced swelling (FIS) of intestinal organoids was performed similar to previously described methods [38, 39]. Briefly, intestinal organoids which had been cultured for 6–8 days were split into non-tissue culture treated 48-well plates (Genesee Scientific) and allowed to grow for 24 hours before drug treatment. Organoids were treated with G418 (20–100 μM) or PTC124 (1–20 μM) for 72 hours prior to FIS measurements. FIS was assessed by treating organoids with 10 μM forskolin and imaging for 5 hours under live cell conditions with brightfield microscopy on a Lionheart FX Automated Microscope (BioTek). FIS was quantified by identifying and normalizing organoid area to T = 0 with Gen5 ImagePrime software (BioTek). Area under the curve (AUC) at T = 300 minutes was calculated to compare statistical significance between treatment groups. All organoids used for swelling quantifications were between passages 1–4.

Statistics. Results are expressed as the mean +/- S.E.M. Differences between groups were determined using either an ANOVA with post-hoc Tukey test or an unpaired t test. A P value of <0.05 was considered significant.

Results

Generation of the G542X mutation

To generate the G542X mutation in mouse Cfr, the CRISPR/Cas9 gene editing system was utilized. Guide RNAs (gRNAs) in proximity to the DNA sequence corresponding to glycine at position 542 in mouse Cfr exon 12 were selected and tested in vitro for their ability to support efficient Cas9 nuclease activity to the DNA region as indicated by the reduction of full length DNA amplicon (Fig 1A). The gRNA that supported the highest Cas9 nuclease activity was injected in one-cell mouse embryos along with a ssODN containing the G542X mutation and either Cas9 mRNA or protein (Fig 1B). DNA from all mice originating from these injected embryos was sequenced. Of 22 mice, 9 (40.9%) had at least one Cfr allele containing the G542X mutation due to homology-directed repair (HDR) with the ssODN, 5 (22.7%) did not have the G542X mutation but had other mutations (insertions/deletions) due to nonhomologous end joining and 8 (34.4%) had no mutation of Cfr in exon 12. One of the founder mice containing the G542X allele was selected to establish and expand the G542X colony.

Cfr expression is reduced and CFTR function is absent in G542X mice

Cfr expression was assessed using qRT-PCR from various tissues of G542X and WT littermates. Cfr expression levels in tissues from G542X mice were significantly reduced compared to WT
expression levels ranging from 2.5±0.5% in the ileum to 28.4±8.6% of WT levels in the lung (Fig 2). Assessment of CFTR function in the airway was performed using NPD measurements in the nasal lumen. Activation of CFTR and the subsequent Cl\(^-\) secretion into the nasal lumen causes a decrease in voltage across the nasal epithelium. Mice with functional CFTR display a decrease in NPD (-14.6±1.6 mV) while G542X mice have a slightly positive NPD (4.38±2.1 mV; Fig 3A) indicating no functional CFTR similar to other CF mouse models [35, 36, 40, 41].

CFTR function in the intestinal epithelium was examined by measuring short-circuit current (ΔI\(_{sc}\)) in intestinal segments from G542X and WT mice before and after activation of CFTR. In WT intestinal segments, a significant increase in short-circuit current is observed following CFTR stimulation. Corresponding segments from G542X mice displayed significantly reduced changes in short-circuit current (Fig 3B), similar to other CF mouse models [36, 42, 43].

G542X mice display characteristic CF manifestations

The most common cause of morbidity in CF mouse models is intestinal obstruction [5, 36, 44, 45]. G542X homozygous mice displayed a reduced survival rate compared to WT littermates (33.3% vs. 97.9%; Fig 4A). Intestinal obstruction was observed in all G542X mice post-mortem.
but not in WT littermates. Another common disease manifestation in CF mouse models is reduced growth, or failure to thrive [31, 36, 43, 46]. G542X homozygous mice demonstrated significantly reduced length compared to sex-matched 6-week old WT littermates (in cm: males 7.6±0.2 vs. 8.4±0.1; females 6.0±0.3 vs. 7.8±0.1; Fig 4B). To assess growth, offspring from G542X heterozygote crosses were weighed at 5 day intervals from 10–40 days of age. G542X homozygous mice displayed significantly reduced weight at all time points compared to sex-matched WT littermates (Fig 4C and 4D).

**G542X CFTR function is restored following pharmacological nonsense mutation readthrough by G418 but not by PTC124**

The ability to produce functional CFTR following treatment with potential therapies is crucial for the G542X mouse model to have full utility as a model of CF nonsense mutations. To examine CFTR function following readthrough, we generated intestinal organoids from G542X homozygous mice. Intestinal organoids are a three-dimensional cell culture model produced from LGR5+ stem cells in the intestinal crypt with budding outgrowths and a hollow lumen [39]. Activation of CFTR by forskolin allows flow of Cl− anions into the intestinal organoid lumen, increasing lumen osmolality, drawing in fluid, and causing organoid FIS. FIS is absent in organoids lacking CFTR function, allowing sensitive detection of CFTR activity. G542X

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**Fig 3. CFTR function in the airway and intestine of G542X mice.** (A) NPD measurements and (B) change in intestinal short circuit current (ΔIsc) measurements from the duodenum, jejunum, and ileum from WT and G542X mice are shown. (*P<0.05 vs. WT. n≥4 per group).  
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**Fig 4. Survival and growth characteristics of G542X mice.** (A) Survival of G542X and WT mice up to 40 days of age. (B) Length of G542X and WT mice at 6 weeks of age. (C, D) Weight of G542X and WT mice sex-matched mice up to 40 days of age. Weight at every age was significantly different between G542X and WT littermates. (n≥10 per group. *P<0.05 vs. WT).  
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organoids were treated for 72 hours with the aminoglycoside G418, a suppressor of nonsense mutations [47, 48]. FIS was examined by imaging organoid swelling over 300 minutes under live cell conditions with brightfield microscopy (Fig 5A). We observed a dose-dependent FIS response to G418-induced readthrough indicating restored CFTR function (normalized percent area at 300 minutes: No G418: 114 ± 1.7; 20 μM: 135 ± 2.3; 50 μM: 160 ± 3.0; 100 μM: 181 ± 1.7, Fig 5B). Statistical significance between treatment groups was compared by calculating area under the curve (AUC) at 300 minutes for each treatment groups (Fig 5C). AUC of organoids treated with G418 were significantly increased compared to non-treated organoids (Fig 5C). S489X organoids, which are non-correctable Cftr-null mutants, did not display FIS following 72 hour treatment with 100 μM G418, confirming that FIS in G542X organoids is due to readthrough of nonsense mutations (Normalized percent area at 300 minutes: 113 ± 0.7, Fig 5B and 5C). Similar to tissues from G542X homozygous mice, cultured intestinal organoids have a reduced amount of Cftr expression compared to those from WT littermates (24.0 ±1.0%) (Fig 5D). However, concurrent with the increase in CFTR function in G418 treated organoids from G542X mice, G418 treatment also significantly increased the amount of detectable Cftr mRNA (Fig 5D). Short term treatment of G542X mice with G418 also resulted in a significant increase in the amount of detectable Cftr mRNA in tissue (Fig 5E). Interestingly, PTC124 treatment of the G542X organoid model did not stimulate detectable FIS at any of the concentrations tested and thus resulted in no significant difference of AUC between PTC124 treated and non-treated organoids (1–20 μM; Fig 6A–6C).

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Fig 5. Intestinal organoids and tissue from G542X mice are used to test G418-mediated nonsense mutation readthrough. Intestinal organoids were incubated with indicated doses of G418, or vehicle for 72 hours prior to swelling with 10 μM forskolin. (A) Representative images at T = 0 and T = 300 minutes of organoid swelling, conditions as indicated. (Scale bar = 100 μm) (B) Total change in area was measured over 300 minutes, and FIS was quantified by normalizing the total organoid area to T = 0. (n = 5 wells of organoids for each treatment, ± SE). (C) AUC at T = 300 for indicated treatment groups. (*P<0.05 compared to untreated G542X). (D) Expression of Cftr in WT and G542X organoids with and without G418 treatment as a percentage of untreated WT organoids. (E) Expression of Cftr in lung from untreated G542X mice or treated with G418 compared to expression from WT mice. (*P<0.05 compared to untreated WT. ^P<0.05 compared to untreated G542X.).
Discussion

Currently there are only two FDA approved therapies that directly target CFTR dysfunction, the primary defect of CF [17, 20]. These therapies are approved to modulate aberrant protein that is produced from the CFTR gene in patients with specific mutations that do not include nonsense mutations. Nonsense mutations typically result in little to no protein function. Reduced mRNA levels, through nonsense-mediated decay (NMD), dramatically decreases the amount of protein produced. In addition, any protein that is synthesized is truncated due to the PTC and, depending on severity of the truncation, leads to significantly limited protein function [49, 50]. Current research efforts to increase the amount of full length protein from nonsense mutations include both inducing readthrough of the PTC and targeting the NMD pathway to increase mRNA levels [49, 50].

Readthrough of PTCs through nonsense suppression can be achieved with aminoglycosides that reduce the efficiency of termination and allow for the insertion of near-cognate tRNA [21]. Promising results in cell and mouse models of nonsense mutations have led to the use of the aminoglycoside gentamicin in patients [14, 21, 24, 51]. In CF, functional improvements in CFTR have been described in CF patients receiving topical application to the nasal mucosa [26] but renal and otic toxicities are a concern with long term treatment. Interestingly, a minor component B1 of gentamicin has been shown to provide effective readthrough activity [52], suggesting that an enrichment of this component of gentamicin or modified aminoglycosides with reduced toxicity may hold promise for the future [23]. A high-throughput screen identified PTC124 as a candidate compound with readthrough ability without the toxicity concerns of aminoglycosides [22]. Despite a lack of readthrough efficacy of PTC124 in specific in vitro reporter systems [53], PTC124 treatment of preclinical models of CF have displayed functional improvements [54]. While some initial patient studies suggested PTC124 efficacy in CF [25, 55], larger studies failed to show significant improvements in CF patients with nonsense mutations in Phase 3 clinical trials [29]. While PTC124 will not be used as a therapy for CF, investigation of the compound is still underway for other nonsense mutation genetic disorders such as Duchenne muscular dystrophy.

There has been recent success in the identification of possible nonsense mutation suppressors in CFTR using high-throughput screens [56–59]. The in vitro tools created in each of these screens...
made the identification of these hits possible. However, a CF animal model to carry out *in vivo* studies to test the efficacy of these hits is not available to the research community. The first *Cftr* mutation made in the mouse was a nonsense mutation created by replacing the normal exon sequence with the premature stop codon, S489X along with selectable markers. The integration of the selectable markers in the exon precludes the production of functional protein [30, 31] and thus serves as a negative control for readthrough strategies. The only current animal model shown to produce functional CFTR following readthrough is a transgenic mouse that expresses human CFTR cDNA containing the G542X mutation driven by a rat Fatty Acid Binding Protein (FABP) promoter that results in intestinal-specific expression [14]. The conditions of CFTR expression in this model differ significantly from endogenous expression and readthrough of G542X can only be assessed in the intestine. In addition, because cDNA was used and there is no intron splicing, the mRNA produced is not susceptible to NMD which limits the interpretation of any *in vivo* studies. Non-endogenous expression levels and/or the absence of NMD of the resulting mRNA may explain why PTC124 displayed functional improvement in the intestine of hCFTR-G542X [54] model but failed to show similar improvements in patients.

In this study, we created a mouse model of CF containing the G542X mutation in the endogenous *Cftr* gene. We utilized the CRISPR/Cas9 gene editing system to create the G542X mutation in one-cell mouse embryos. This is the first report of *Cftr* CRISPR/Cas9 editing to create a mouse model. As previously reported, the use of CRISPR/Cas9 significantly reduces the time of gene editing in embryos compared to embryonic stem cells [60]. In addition, this gene editing system is highly efficient in the creation of the desired mutation. Traditional gene editing in embryonic stem (ES) cells typically produced 1–5% of ES colonies harboring the correctly edited gene and could take 1–2 years to produce the targeting construct and correctly targeted founder mice. With CRISPR/Cas9, 40.9% of founder mice contained the G542X mutation and were produced in as few as 3 months. One disadvantage in utilizing CRISPR/Cas9 gene editing is the possible creation of double-strand breaks in similar sequences to the target which can create off-target effects [61]. A low incidence of off-target mutations has been identified in mice created using CRISPR/Cas9 [62, 63]. While off-target mutations can occur, subsequent backcrosses to a parental strain (e.g., C57Bl/6) can remove any unintended consequences from off-target mutations.

Mice homozygous for the G542X mutation demonstrate reduced *Cftr* expression throughout the body. While our analysis cannot rule out that a truncated form of CFTR protein is produced, there is a clear absence of CFTR function in the airway and intestine of these mice. The G542X mice also display severe CF manifestations, including reduced growth, and reduced survival due to intestinal obstruction. In this model, mRNA levels can be increased and CFTR function can be corrected through readthrough by G418. Interestingly, the utilization of PTC124 in the intestinal organoid model did not demonstrate any improvement in CFTR function. This is consistent with studies that show no significant improvement in CF patients chronically administered PTC124. All of these findings confirm that this G542X mouse model can be utilized to test the efficacy of nonsense mutation therapies.

The G542X mouse model also provides an unlimited source of primary cells to potentially identify new therapies for nonsense mutations. For example, the prominent intestinal organoid FIS phenotype, which indicates CFTR function, has the potential to be used for high-throughput screens similar to those currently utilizing CF patient samples [39, 64]. The high degree of similarity between organoids and tissue *in vivo* may increase the odds of identifying translationally beneficial compounds in a high-throughput screen compared to current screens using transfected cell lines or *in vitro* reporter assays.

In conclusion, this is the first study to generate a mouse model containing CRISPR/Cas9 edited *Cftr* and the first CF animal model that can be utilized to assess whole body efficacy of
nonsense mutation directed therapies. With several recent screens identifying potential PTC readthrough compounds, the G542X mouse model can be utilized to prioritize which compounds to test in a clinical trial that may be beneficial to CF patients. The ability to analyze in vivo effectiveness of current and future potential nonsense mutation therapies will not only increase our understanding of how these therapies work on a basic level but lead to treatments for CF patients with nonsense mutations.

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