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Functional Repair of CFTR by CRISPR/Cas9 in Intestinal Stem Cell Organoids of Cystic Fibrosis Patients

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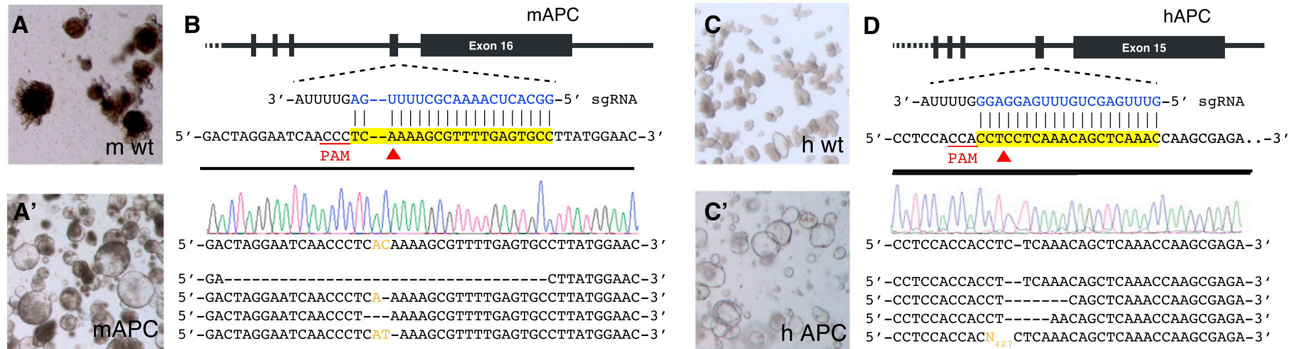
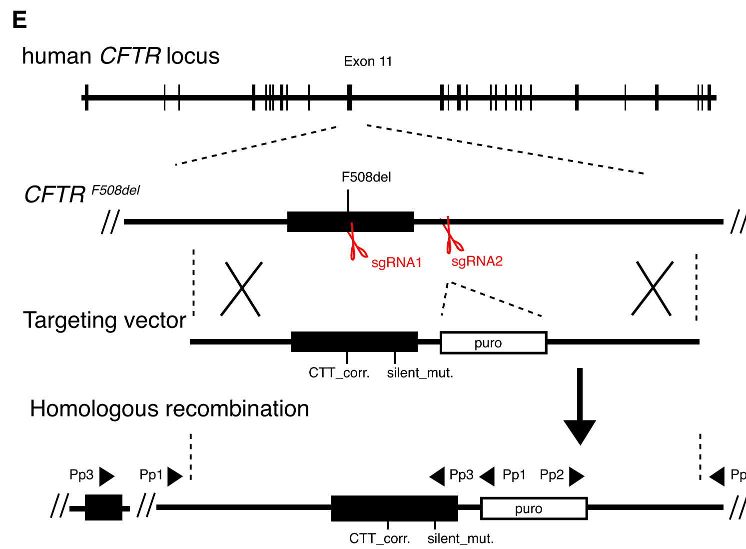
SUMMARY

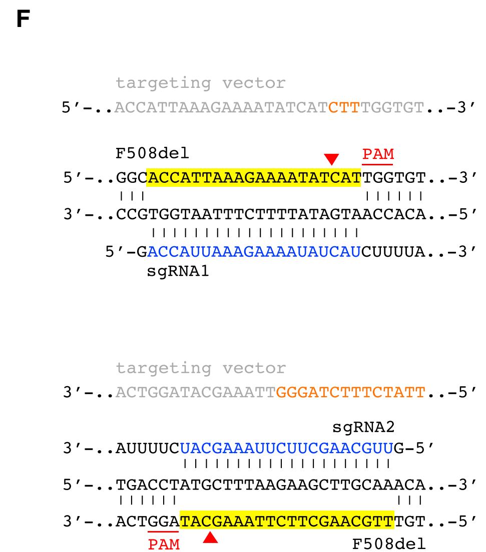
Single murine and human intestinal stem cells can be expanded in culture over long time periods as genetically and phenotypically stable epithelial organoids. Increased cAMP levels induce rapid swelling of such organoids by opening the cystic fibrosis transmembrane conductor receptor (CFTR). This response is lost in organoids derived from cystic fibrosis (CF) patients. Here we use the CRISPR/Cas9 genome editing system to correct the CFTR locus by homologous recombination in cultured intestinal stem cells of CF patients. The corrected allele is expressed and fully functional as measured in clonally expanded organoids. This study provides proof of concept for gene correction by homologous recombination in primary adult stem cells derived from patients with a single-gene hereditary defect.

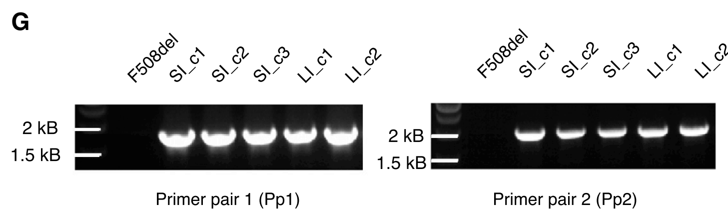
We have previously described a culture system that allows apparently indefinite in vitro expansion (for >1 year) of single murine Lgr5$^+$ intestinal stem cells into a 3D small intestinal epithelium (Sato et al., 2009). A crucial ingredient is the Wnt agonistic R-spondin1, a ligand of Lgr5 (Carmon et al., 2011; de Lau et al., 2011). Intestinal organoids or "miniguts" comprise nearly intact physiology; self-renewing Lgr5$^+$ stem cells and the niche-supporting Paneth cells are located in a domain that resembles the crypt, and enterocytes as well as goblet and enteroendocrine cells move upward to build a villus-like domain that lines the central lumen. Minor adaptation of this culture condition allowed us to develop similar types of organoid cultures for colon, stomach, liver, and pancreas using mouse and human tissues (Barker et al., 2010; Huch et al., 2013b, 2013c; Jung et al., 2011; Sato et al., 2011). Successful transplantation of clonal organoids derived from single Lgr5$^+$ stem cells into damaged tissue has been demonstrated for mouse colon and liver, making the organoid system a promising tool for adult stem cell/gene therapy (Huch et al., 2013a; Yui et al., 2012). Recently, several groups have demonstrated the use of the CRISPR/Cas9 system for genome engineering in various species (Chang et al., 2013; Cho et al., 2013; Cong et al., 2013; Friedland et al., 2013; Hou et al., 2013; Hwang et al., 2013; Jinek et al., 2013; Li et al., 2013; Mali et al., 2013; Nekrasov et al., 2013; Shen et al., 2013; Wang et al., 2013; Xiao et al., 2013; Yu et al., 2013). The system utilizes the type II prokaryotic CRISPR/Cas9 adaptive immune system and targets the Cas9 nuclease by a 20 nt guide sequence cloned upstream of a 5′-NGG "protospacer adjacent motif" (PAM) (Jinek et al., 2012). The induced site-specific double-strand breaks are repaired either by nonhomologous end-joining (NHEJ) to yield indels (Barnes, 2001) or by homologous recombination (HR) if homologous donor templates are available (van den Bosch et al., 2002), thereby enhancing the efficiency of HR-based gene targeting (Bibikova et al., 2003; Porteus and Carroll, 2005; Thomas and Capecchi, 1987; Urnov et al., 2005). The high efficiency and simple design principle of the CRISPR/Cas9 system prompted us to evaluate its use for gene manipulation of adult stem cells in Lgr5/R-spondin-based organoid cultures.

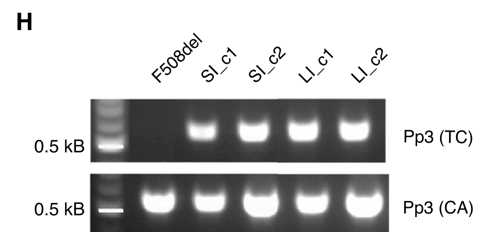
We first optimized the CRISPR/Cas9 system by targeting the murine *APC* locus in adult intestinal stem cells. The optimized protocol involves culturing intestinal organoids in Wnt-conditioned media, trypsinization to obtain a single cell suspension, and Lipofectamine-mediated transfection with plasmids expressing Cas9 and sgRNAs targeting *APC* (Figures S1A and S1B available online) (Schwank et al., 2013). Of note, only Lgr5$^+$ stem cells—and none of the other epithelial cell types—will grow out in a clonal fashion into secondary organoids in culture (Sato et al., 2009, 2011). As APC is a negative regulator of the Wnt pathway, stem cells in which both *APC* alleles are inactivated will grow out in the absence of the normally essential

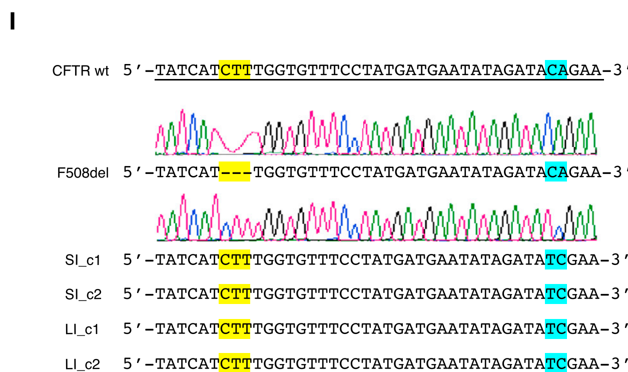


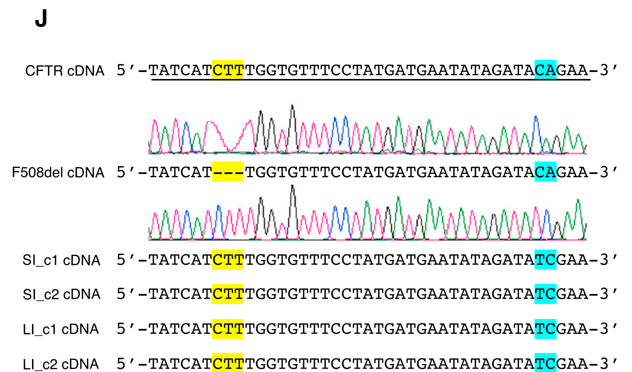












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Wnt agonist R-spondin1. Two weeks after seeding transfected single cells, multiple organoids grew out from the pool of cells cotransfected with each of five different sgRNAs. In contrast to budding wild-type organoids, selected *APC* mutant organoids showed a cystic morphology (Figures 1A and 1A′), and sequencing of isolated clones confirmed mutations in the targeted *APC* locus (Figure 1B, Figure S1C). No organoids grew in control transfections without the specific sgRNAs. Double-targeting of the two negative Wnt regulators RNF43 and its homolog Znrf3 (Hao et al., 2012; Koo et al., 2012) also resulted in surviving organoids with frameshifts in both targeted loci (Figure S1D), demonstrating the possibility to efficiently generate four-allele knockout organoids in a single transfection. We then tested the CRISPR/Cas9 system on human adult intestinal stem cells by targeting the *APC* locus. As human intestinal stem cells in culture require additional Wnt for self-renewal and expansion (Jung et al., 2011; Sato et al., 2011), transfected stem cells were seeded in medium lacking both Wnt and R-spondin. Organoids only grew out from the pool of cells cotransfected with the specific sgRNA, and selected clones showed a cystic morphology (Figure 1C′). Sequencing confirmed mutations in the targeted region (Figure 1D), demonstrating the potential of the CRISPR/Cas9 system for genome editing of adult human stem cells in primary intestinal organoids.

To investigate the possibility of gene correction in adult stem cells using CRISPR/Cas9, we focused on the cystic fibrosis transmembrane conductor receptor (CFTR) in primary cultured small intestinal (SI) and large intestinal (LI) stem cells. *CFTR* encodes an anion channel essential for fluid and electrolyte homeostasis of epithelia. Mutations in this receptor cause cystic fibrosis (CF) (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989), a disease that leads to the accumulation of viscous mucus in the pulmonary and gastrointestinal tract and to a current median life expectancy of approximately 40 years (Ratjen and Döring, 2003). We established SI and LI organoids from two different pediatric CF patients. Both patients were homozygous for the most common *CFTR* mutation, a deletion of phenylalanine at position 508 (CFTR F508 del) in exon 11, which causes misfolding, endoplasmic reticulum retention, and early degradation of the CFTR protein (Cheng et al., 1990). To first demonstrate the loss of the CFTR function, we performed the previously established forskolin-induced swelling assay. Forskolin activates CFTR by raising the amount of intracellular cyclic AMP, leading to fluid secretion into the lumen and swelling of organoids (Dekkers et al., 2013). Unlike wild-type organoids, the CFTR F508 del patient organoids did not expand their surface area upon forskolin treatment (Figures 2A, 2C, and 2D), confirming loss of function of CFTR as published previously.

We then transfected the patient organoids independently with two different sgRNAs targeting either *CFTR* exon 11 or intron 11, together with a donor plasmid encoding wild-type CFTR sequences (Figure 1E). Downstream of the corrected F508 del mutation, we introduced a silent mutation into the donor sequence enabling allele-specific PCR testing. Within the intronic sequence, we incorporated a puromycin resistance cassette (Figure 1E). The sgRNAs were designed to cut the genomic CFTR sequence, but not the homologous sequence within the targeting vector (Figure 1F). After transfection single cells were plated, and organoids derived from puromycin-resistant individual stem cells were selected and tested for site-specific integration of the donor plasmid by PCR with primers outside of the 5′ and 3′ homology arms and within the puromycin selection cassette (Figure 1E). For both patients, we retrieved several organoid clones with each of the two sgRNAs (Table S1; selected clones are shown in Figure 1G). We confirmed site-specific knockin events and correction of the F508 del allele by sequencing the recombined allele (Figure 1I). Note that sequencing the second allele revealed heterozygous CFTR repair in the majority of clones (Table S1). Transfection with sgRNA2, which induces a double-strand break 203 base pairs (bp) downstream of the F508 del mutation also generated a clone with an anecdotal knockin event where the recombination appeared downstream of the mutation and repair was not achieved (SI_c3 in Figure 1G). To validate expression of the corrected allele, we performed RT-PCR using a forward primer in exon 10 and an allele-specific reverse primer that binds

Figure 1. CRISPR/Cas9-Mediated Genome Editing in Adult Stem Cells

(A–D) Generation of indels in the mouse and human *APC* locus. (A) Wild-type mouse intestinal organoids in complete growth medium, and (A′) *APC* mutant mouse intestinal organoids generated with the CRISP/Cas9 system and selected in medium without R-spondin. Note that organoids change their morphology from budding structures (wild-type) to cystic structures (APC). (B) Schematic of the targeted region of the mouse *APC* locus, and sequences of five mutant alleles from selected clones. Regions of the sgRNA complementary to the protospacer (yellow) are shown in blue. Red arrowheads indicate cleavage sites. (C) Wild-type human intestinal organoids in complete growth medium, and (C′) *APC* mutant human intestinal organoids selected in medium without Wnt and R-spondin. (D) Schematic of the targeted region of the human *APC* locus, and sequences of five mutant alleles from selected clones.

(E–J) Correction of the human *CFTR F508del* allele by induced homologous recombination. (E) Strategy of the genome modification using CRISPR/Cas9 to induce double-strand breaks in the *CFTR* locus, and a template for homology directed repair. Top line, structure of the *CFTR* gene. Black boxes illustrate exons, and thin strokes illustrate introns. Red scissors show cleavage sites of sgRNA1 and sgRNA2, and white box in the targeting vector indicates the puromycin selection cassette. A 2 bp silent mutation is introduced downstream of the CTT F508del correction and allows allele-specific PCR testing. Pp1, Pp2, and Pp3 illustrate PCR primer pairs. (F) Schematic representation of base pairing of the targeting locus with sgRNA1 (upper panel) and sgRNA2 (lower panel). Top lines illustrate the corresponding sequences in the targeting vector. Nonmatching bases are shown in orange and are based on the F508del correction (CTT addition) and insertion of the selection cassette, respectively. (G) PCR analysis showing insertion of the targeting vector by homologous recombination. Positions of Pp1 and Pp2 primers-pairs are illustrated in (E). SI_c1: clone derived from SI organoids of patient 1 corrected by cleavage with sgRNA1. SI_c2: clone derived from SI organoids of patient 1 corrected by cleavage with sgRNA2. SI_c3: same as SI_c2, but integration of the selection cassette did not result in F508 del correction. LI_c1: clone derived from LI organoids of patient 2, corrected by cleavage with sgRNA1. LI_c2: clone derived from LI organoids of patient 2, corrected by cleavage with sgRNA2. (H) RT-PCR analysis of the CFTR cDNA with primers specific for the corrected allele Pp3(TC) and the uncorrected allele Pp3(CA), respectively. Pp3 forward primer is located in exon 10. (I and J) PCR amplification products of the corrected alleles (from G and H) were sequenced. This revealed correction of the F508 del mutation in the genomic locus (I) and cDNA (J). Note that the clones shown here are heterozygous for the corrected allele and retained one copy of the mutant allele (data not shown).

See also Figure S1, Table S1, and Table S2.

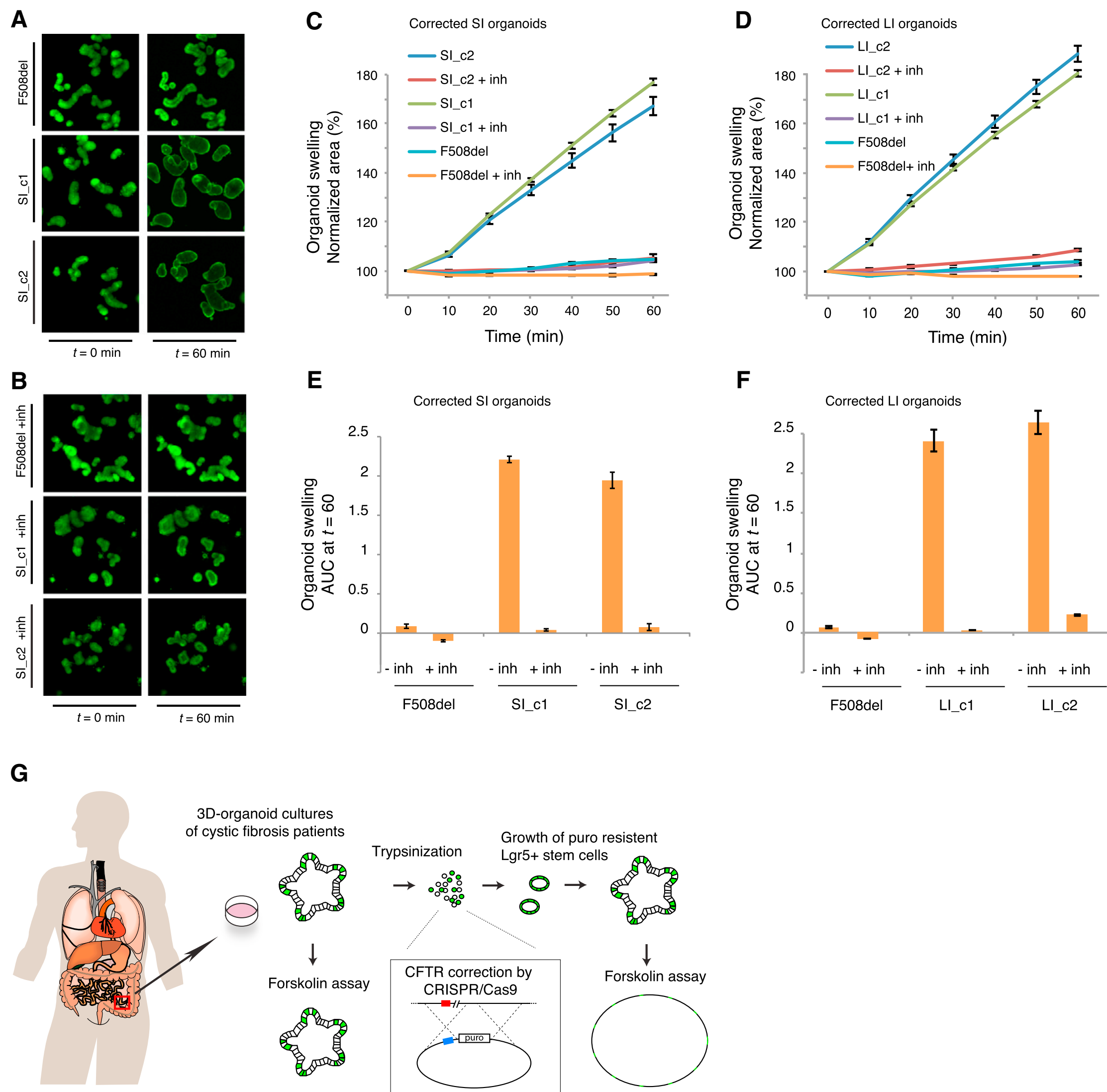

Figure 2. Functional Analysis of the Restored CFTR Function in Corrected Organoids

(A and B) Confocal images of calcein-green-labeled and forskolin-stimulated SI organoids (A) without and (B) in the presence of a chemical CFTR inhibitor. SI_c1, SI_c2: clones derived from SI organoids corrected by cleavage with sgRNA1 and sgRNA2, respectively. F508 del: uncorrected control organoids of the corresponding patient. t = 0 min, t = 60 min indicate time points after forskolin induction.

(C and D) Quantification of organoid swelling of corrected SI organoid clones (C) and LI organoid clones (D). The total organoid surface area is normalized to t_0 min and measured in three independent wells. Error bars indicate the standard error of the mean (SEM). inh, chemical CFTR inhibitor.

(E and F) Forskolin-induced swelling expressed as the absolute area under the curve calculated from (C) and (D), respectively (baseline = 100%, t = 60 min). Error bars indicate SEM.

(G) Schematic illustration of the gene correction protocol. Stem cells are labeled in green. Note that after transfection only stem cells that integrated the selection cassette can grow out and form new organoids.

See also Movie S1.

exclusively to the introduced silent mutations in exon 11. Expression of the repaired allele was absent in uncorrected control organoids and detected in all transgenic clones (Figures 1H and 1J). RT-PCR with a reverse primer specific for the uncorrected allele confirms heterozygousity of the knockin events.

It has been reported that sgRNAs can potentially tolerate mismatches in the 20 bp protospacer target sequence, which can lead to the generation of undesirable "off-target" indels (Hsu et al., 2013; Mali et al., 2013; Pattanayak et al., 2013). To assess off-target effects of the CRISPR/Cas9 system in our adult primary stem cell system, we computationally identified possible off-target sites for each of the two sgRNAs (sequences with one to three mismatches to the protospacer followed by the NGG-PAM motif). We identified 29 potential off-target sites for sgRNA1, of which 25 were sequenced and analyzed in an individual clone. Only one site contained a 4 bp insertion in the protospacer sequence (Table S2). Notably, the mutation was heterozygous and located within an intron, making phenotypic consequences highly unlikely. For sgRNA2, we identified and sequenced 17 off-target sites in one clone, and no mutations

were found (Table S2). Also, when protospacer-homology regions with 4 mismatches (10 sites for sgRNA1 and 8 sites for sgRNA2) were analyzed, we did not find any indels (Table S2), confirming previous studies that suggest off-target effects to be limited to sites with only one to three mismatches (Mali et al., 2013). Our results therefore demonstrated high specificity of the CRISPR/Cas9 system in adult stem cells.

To assess whether the CFTR function in corrected organoids was restored, we performed the forskolin assay with transgenic lines. By live-cell microscopy, we observed rapid expansion of the organoid surface area in the corrected organoids, whereas swelling was absent in untransfected control organoids (Figure 2A, Movie S1). Quantification of swelling by automated image analysis demonstrated a relative increase of the total organoid surface area to 177% (±1.4 SEM) and 167% (±3.8 SEM) for two corrected SI organoid clones (Figures 2C and 2E), and to 187% (±3 SEM) and 180% (±1.5 SEM) for two corrected LI organoid clones (Figures 2D and 2F). These numbers are comparable to forskolin-induced surface area increase of wild-type organoids and exceed CFTR rescue capacities obtained with chemical correctors (Dekkers et al., 2013). Untransfected F508 del organoids increased only marginally in surface area (Figures 2C–2F), which is consistent with very limited residual CFTR function of the F508 del allele (Dekkers et al., 2013). We next tested whether the forskolin-induced swelling of the corrected organoids was sensitive to chemical inhibition of CFTR by CFTRinh-172 (Thiagarajah et al., 2004). Indeed, forskolin-induced swelling was fully abolished in presence of the inhibitor (Figures 2C–2F, Movie S1). Together, these data demonstrated that the corrected F508 del allele was fully functional and was able to rescue the CFTR phenotype in organoids.

In summary, we have isolated and expanded adult intestinal stem cells from two CF patients, corrected the mutant F508 del allele using the CRISPR/Cas9 mediated homologous recombination, and demonstrated functionality of the corrected allele in the organoid system (Figure 2G). Together with previous studies, in which in vitro expanded organoids were successfully transplanted into colons of mice (Yui et al., 2012), this work provides a potential strategy for future gene therapy in patients. Although given its multiorgan involvement CF does not appear to be a prime candidate for clinical application of adult stem cell gene therapy, this approach may present a safe complement to induced-pluripotent-stem-cell-based approaches, and in the future it could be applied to different single-gene hereditary defects. The advantage of combining HR-based gene correction strategies with organoid culture technology rests in the possibility of clonal expansion of single adult patient stem cells and the selection of recombinant clonal organoid cultures harboring the desired, exact genetic change.

SUPPLEMENTAL INFORMATION

Supplemental Information for this article includes Supplemental Experimental Procedures, one figure, two tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.stem.2013.11.002.

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