[Frontiers in Bioscience 13, 2989-2999, January 1, 2008]

CFTR gene targeting in mouse embryonic stem cells mediated by *S*mall *F*ragment *H*omologous *R*eplacement (SFHR)

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TABLE OF CONTENTS

1. ABSTRACT

Different gene targeting approaches have been developed to modify endogenous genomic DNA in both human and mouse cells. Briefly, the process involves the targeting of a specific mutation *in situ* leading to the gene correction and the restoration of a normal gene function. Most of these protocols with therapeutic potential are oligonucleotide based, and rely on endogenous enzymatic pathways. One gene targeting approach, "Small Fragment Homologous Replacement (SFHR)", has been found to be effective in modifying genomic DNA. This approach uses small DNA fragments (SDF) to target specific genomic loci and induce sequence and subsequent phenotypic alterations. This study shows that SFHR can stably introduce a 3-bp deletion (deltaF508, the most frequent cystic fibrosis (CF) mutation) into the *Cftr* (CF Transmembrane Conductance Regulator) locus in the mouse embryonic stem (ES) cell genome. After transfection of deltaF508-SDF into murine ES cells, SFHR-mediated modification was evaluated at the molecular levels on DNA and mRNA obtained from transfected ES cells. About 12% of transcript corresponding to deleted allele was detected, while 60% of the electroporated cells completely last any measurable CFTR-dependent chloride efflux The data indicate that the SFHR technique can be used to effectively target and modify genomic sequences in ES cells. Once the SFHR-modified ES cells differentiate into different cell lineages they can be useful for elucidating tissue-specific gene function and for the development of transplantation-based cellular and therapeutic protocols.

2. INTRODUCTION

Oligonucleotides-mediated gene modification in eukaryotic cells has the potential to correct or introduce specific mutations in the genome while maintaining the integrity of the target gene. These gene targeting strategies will retain the relationship between the protein coding sequences and the gene-specific regulatory elements and make it possible to have a long term, tissue specific, and genetically heritable expression of the modified sequences.

We and others have shown that a gene targeting approach, called Small Fragment Homologous Replacement (SFHR), efficiently introduces chromosomal gene alterations into mammalian cells either "*in vitro*" and "*in vivo*" (1-8).

SFHR employs small DNA fragments (SDF) that are homologous to the genomic target to catalyze intracellular enzymatic mechanisms that mediate

homologous exchange (9-15). SDFs, once introduced into the nuclei, facilitate homologous exchange between incoming SDF sequences and endogenous sequences that ultimately result in genotypic and phenotypic changes (16-17). The process can lead to different genomic alterations that include single base substitutions as well as concomitant insertion or deletion of multiple bases. These SFHR-mediated modifications have been observed within the CFTR gene, the human ß-globin gene (Hß-G), the mouse dystrophin (*mdx*) gene, the human SMN (Survival Motor Neuron) gene, and the murine *DNA-PKcs* gene, responsible for SCID disease (5,6,8,18-21). These findings suggests that SFHR has a broad range of utility both in terms of the target gene and of the cell type.

SFHR gene modification frequency is estimated to be in the range of 1-10% *in vitro* (5) and appears to be influenced by the method with which the DNA is delivered. Recent studies suggest that this efficiency can be significantly increased by nucleofection or by direct nuclear injection of the SDF (8, 20,21). However, the enzymatic mechanisms underlying SFHR have yet to be elucidated (22).

This study shows that SFHR is able to stably modify the *Cftr* locus in the genome of mouse embryonic stem (ES) cells and introduce a 3-bp deletion specifically within the mouse equivalent of human exon 10. SFHR-mediated modification was evaluated at both DNA and RNA levels, and confirmed by functional physiological studies, which revealed a conspicuous reduction of CFTR channel activity in modified ES cells. SFHR application to modify the ES cell genome has important implications for cell and gene therapy in general. ES cells have the ability to differentiate into a variety of tissues that could potentially be used to repair organ damage caused by disease pathology (23-26). Furthermore, this novel methodology facilitates the generation of "*in vitro"* modified tissues that can be used as models for genetic diseases and to analyze gene function in specific tissues.

3. MATERIAL AND METHODS

3.1. SDF preparation

SDF (783-bp) containing the ΔF508 mutation and a unique KpnI restriction site was synthesized by PCR amplification using primers mCF1 and mCF15, (Figure1A) as described previously (2). The KpnI site described for this locus is absent within murine genomic DNA and can be used as a marker to assess SFHR-mediated modification. The single base modification was introduced into the ΔF508-SDF by a modified megaprimer protocol (27). The resultant SDF cloned in a plasmid, was used for large-scale SDF production. Before transfection the SDF was used, always gel and ethanol purified (DNA gel extraction kit; Millipore, Bedford, MA). Briefly, preparative amounts of ΔF508-SDF were generated in a total volume of 50 µl, containing 1X PCR buffer, 1.5 U of *Pfu* DNA polymerase, 20 pmol of each primer, 2 ng of plasmide (ΔF508-SDF) genomic DNA with an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation; 94°C for 30 sec; annealing at 61°C for 30 sec, and extension at 72°C for 1 min with a final extension for 8 min at 72°C.

3.2. Cell culture

ES-D3 cells were obtained from the ATCC and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% FCS and 1000 U/ml LIF (ESGRO, Chemicon Inc., CA, USA; http://www.esgro-lif.com) at 37°C under 5% CO_2. The ES cells were adapted to grow off feeders onto gelatin-coated tissue culture dishes, to avoid obscuring the interpretation of the results. The differentiated state of ES cells was routinely monitored by assaying for the presence of alkaline phosphatase. Under these growth conditions the ES-D3 cells form colonies of 23-25 cells within four days of seeding on glass coverslips.

3.3. ES nucleofection

Transfection of the D3-ES cells was achieved by electroporation (nucleofection) with the AMAXA Nucleofection System according to the mouse ES cell protocol developed by the manufacturer (AMAXA Biosystems, Köln, Germany). Approximately, $1.5x10^6$ cells were trypsinized, washed in Phosphate Buffer Saline (PBS, Cambrex, NJ, USA) and resuspended in 100 µl of Mouse ES Cell Nucleofector solution (AMAXA Biosystems). ΔF508-SDF was transfected at different concentrations: 800, 1600, and 2400 µg equivalent to ~ $6.4\ x10^5$, $1.2\ x10^6$, $1.9\ x10^6$ SDF molecules per cell, respectively. SDF concentration was determined spectrophotometrically (ND-1000, Nanodrop Spectrophotometer, Wilmington, Delaware USA). Program A30 was used in conjunction with the Mouse ES Cell Nucleofector solution to transfect the ΔF508-SDF into D3 cells. After electroporation, cells were plated immediately, expanded, and after five days harvested for analysis. As a control, D3-ES cells were transfected with a 498 bp SDF homologous to *Smn* gene (10^7 SDF per cell) (8).

3.4. DNA and RNA isolation

DNA was isolated using phenol-chloroform. RNA was extracted with Trizol (Gibco BRL, Gaithersburg, USA), DNAse treated, and then resuspended in DEPC water. All nucleic acids were quantified spectrophotometrically (ND-1000 Nanodrop Spectrophotometer). The mRNA was reverse-transcribed into cDNA according to the manufacturer's instructions (High-Capacity cDNA Archive Kit Applied Biosystems, Foster City, CA USA; http://www.appliedbiosytems.com). Briefly, a 50 µl aliquot of 2X RT Master Mix (2X RT buffer, 2X dNTP mixture, 2X random primers, 5U of MultiScribe RT) was added to tubes containing 50 µl of RNA (500ng-1500ng) and then incubated for 10min at 25°C and 2 hours at 37°C.

3.5. PCR amplification of DNA and mRNA

Allele-specific PCR (AS-PCR) protocols were used for both DNA and mRNA analysis of the transfected

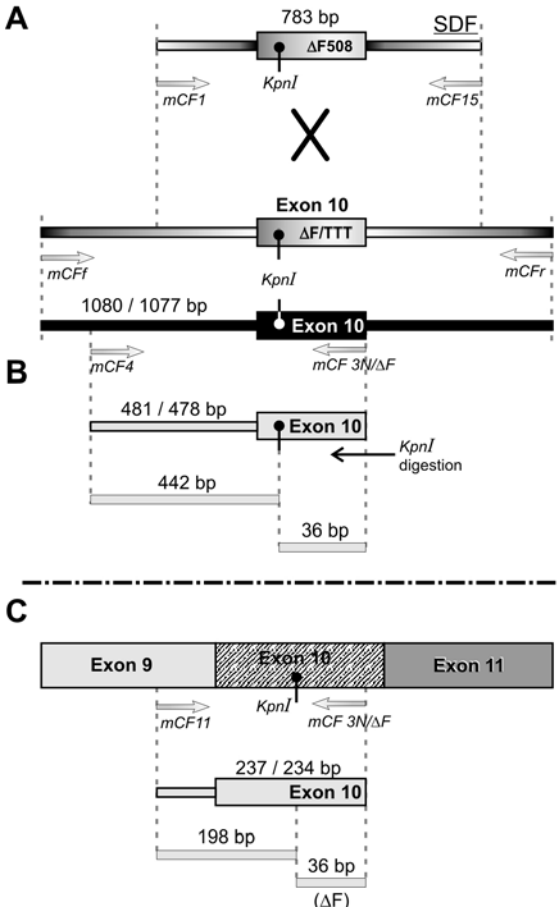

Figure 1. Schematic of small DNA fragment (SDF) generation and PCR analysis of SFHR A. SDF (783bp) containing the ΔF508 mutation and a KpnI restriction enzyme cleavage site was synthesized using primers mCF1 and mCF15, localized within introns 9 and 10 of *Cftr* gene respectively (2). The unique KpnI site is a secondary marker for assessment of SFHR-mediated modification. B. Analysis of genomic DNA using two successive rounds of PCR amplification. The first round of PCR used primers (mCFf/mCFr) were located outside the region of homology defined by the SDF and resulted in a 1080- or 1077-bp amplicon (wild tyoe of ΔF508, respectively). The second round of amplification used the amplicon generated in the first round as a template and allele specific primers (mCF4/mCF3N or mCF4/mCFΔF for wild type and ΔF508 sequence respectively). The 478-bp fragment was then digested with KpnI to determine whether the 442- and 36-bp restriction fragments, indicating SFHR mediated replacement, were present. C. Allele specific RT-PCR for analysis of transfected ES cells using primers located within exon 9 (mCF11) and exon 10 (mCF ΔF or mCF3N, wild type and ΔF508 respectively). The 237- or 234-bp amplicon was then digested with KpnI to assay for restriction fragments of 198-bp and 36-bp indicating expression of SDF-derived sequences.

ES cells. The ΔF508 allele was detected by a two-step PCR amplification performed on genomic DNA from transfected and untransfected cells. The first step used primers that were located outside the region of homology defined by the ΔF508-SDF (mCFf: 5'-ttaaagatgaaagcaaattttcata-3' and mCFr: 5'-ATTCACTGACCCACCCACTC-3' and produced a 1080/1077 bp amplicon for both wild type and deleted sequences, respectively (Figure 1B). PCR was performed in a total volume of 50 µL containing 200 mM each of four dNTPs, 2.5 mM $MgCl_2$, 0.25 U Taq polymerase, 20 pmol of each primer, and 150 ng of genomic DNA. The initial denaturation 95°C for 5 min was followed by 35cycles of denaturation: 94°C for 1 min, annealing: 58°C for 1 min, and extension: 72°C for 1 min, with afinal extension step of 72°C for 7 min. Amplicons were gel-purified by spin columns (Millipore, MA, USA, http://www.millipore.com) and used as the template for a second round of amplification . The second step involved a nested PCR amplification in which the primer mCF4: 5'-cacactcatgtagttagagcatagg-3' was located outside of SDF paired with the allele-specific primers mCF3N: 5'-ATCATAGGAAACACCAAA-3' or mCF3ΔF: 5'-ATCATAGGAAACACCGAT -3' (wild type or mutant, respectively) (Figure 1B). PCR was carried out in a total volume of 30 µL of reaction solution described above, using 15 pmol of each primer. Amplification was for 35 cycles as follows; denaturation: 94°C for 30 sec, annealing: 59°C for 30 sec, and extension: 72°C for 30 sec with a final extension cycle at 72°C for 7 min.

Digestion of the 478-bp analytical PCR fragment with KpnI produces two restriction fragments (442-bp and 36-bp) for the ΔF508-specific amplification, while there will be no digestion of the wtCFTR-specific 481-bp . The KpnI restriction site is used as a secondary marker of an SDF-induced homologous exchange. After KpnI digestion, the sample was banded on a 6% polyacrylamide gel.

For analysis of CFTR mRNA, one primer was in exon 9 (mCF11) and was paired with either mCF3N or mCF3ΔF (wild-type and deltaF508, respectively) localized within exon 10(figure 1C). The 234-bp ΔF508-specific amplicon yields a 198 and a36 bp fragment following KpnI digestion if the SDF-derived sequences have been appropriately introduced into the genomic DNA and correctly transcribed into mRNA (2).

3.6. Cloning of PCR amplicons

AS-PCR products from mRNA-derived cDNA were cloned into the pCR 2.1 of the TA cloning system following manufacturer's instructions (InVitrogen, Carlsbad, CA, USA). Each bacterial clone was grown in LB (100 µg/ml ampicillin) at 37°C. The cellular pellet was lysed by heating at 94°C and then directly amplified in 50 µL total volume of 200 mM each of four dNTPs, 2.5 mM $MgCl_2$, 0.25 U Taq polymerase, 20 pmol of each primer, DMSO 1,8 µL with primers M13 forward (5'-GTAAAACGACGGCCAGT-3') and M13 reverse (5'-CAGGAAACAGCTATGAC-3') primers using the following amplification conditions: 35 cycles of; denaturation: 94°C for 30 sec, annealing: 55°C for 30 sec, and extension: 72°C for 30 sec with a 7 min extension on the last cycle. PCR amplicons were then digested with KpnI and run on a 2.5% agarose gel. Each clone was also

sequenced to verify the presence of the deletion and the KpnI restriction site.

3.7. Real-time PCR analysis of gene expression

Real time RT-PCR was performed using a TaqMAN ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA USA) using the following conditions: 2 min incubation at 50°C (for optimal AmpErase UNG activity) followed by 10 min incubation at 95°C (for deactivation of AmpErase UNG activity and activation of AmpliTaq Gold). Samples were then amplified for 40 cycles of denaturation: 15 sec at 95°C and annealing/extension: 1 min at 60°C. Primers were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, CA). Wild type and mutant alleles were differentiated using MGB probes. CFTR forward primer:5'-TTTCTTGGATTATGCCGGGTACT-3'; CFTR reverse primer: 5'-GCAAGCTTTGACAACACTCTTATATCTG-3';CFTR wt-specific MGB probe: 5'-FAM-TATCATCTTTGGTGTTTCC-3';CFTR ΔF508–specific MGB probe: 5'-VIC-ATATCATCGGTGTTTTCCTAT-3'. A commercially available endogenous gene, phosphoglycerate kinase 1 gene (Pgk1: Mm 00435617_m1) was used as a reference for the TaqMan assay. This reference gene is assumed to be constant in both transfected and untransfected samples and was used to normalize the amount of cDNA added per sample. A comparative C_T method was used to quantify relative gene expression. All PCR reactions were performed in triplicate. Results are expressed as relative levels of the ΔF508 allele mRNA compared to wtCFTR expression (represented as a 1X expression of the CFTR gene). The samples were calibrated against a sample of untransfected cells that was analyzed on every assay plate with the transfected cells.

3.8. Fluorescence chloride efflux measurements

Chloride efflux was measured using the Cl^- sensitive dye MQAE as previously reported (27). Cells seeded on 0.1% gelatin coated glass coverslips, were loaded overnight in culture medium containing 5 mM MQAE at 37°C in a CO_2 incubator. After several washes, the coverslips with cells was inserted into a perfusion cuvette (28). A restricted area of the cells (1.8 x 2.5 mm) on the coverslips was excited. Fluorescence was recorded with a Cary Eclipse Varian spectrofluorometer using 360 nm (bandwidth 10 nm) as excitation wavelength and 450 nm (bandwidth 10 nm) as emission wavelength. All experiments were performed at 37°C in HEPES-buffered bicarbonate-free media (Cl^- medium (in millimolar): NaCl 135, KCl 3, $CaCl_2$ 1.8, $MgSO_4$ 0.8, HEPES 20, KH_2PO_4 1, glucose 11, and Nitrate-medium: $NaNO_3$ 135, KNO_3 3, $MgSO_4$ 0.8, KH_2PO_4 1, HEPES 20, $CaNO_3$ 5, glucose 11).

3.9. Video Imaging Experiments Cl- measurements

In some experiments the Cl- efflux was detected by simultaneous fluorescence measurements from different regions of individual colonies of cells using a video imaging system. Coverslips with dye-loaded cells (by overnight incubation in 5 mM MQAE) were mounted in an open-topped perfusion chamber (Series 20, Warner Instrument Corp, Hamden, CT) and placed on the heated stage of a Nikon TE200 inverted microscope. Cells were excited at 370 nm for 100 ms through a 40 (NA 1.4) oil immersion objective. The 370 nm excitation wavelengths were generated by a monochromator (DeltaRam V, PTI) placed in the path of a xenon light source. Fluorescente images (emission collected at 450 nm) were captured by a Hamamatsu ORCA ER CCD camera every four seconds to minimize photobleaching and processed by the Metafluor software (Universal Imaging, West Chester, PA) to yield background-corrected pseudocolour images reflecting the 370 nm fluorescence. Contributions of autofluorescence were measured and found to be negligible. To measure the CFTR-dependent chloride efflux rate across the cell membrane by the two techniques described above, the perfusion medium was changed to a medium in which chloride was substituted with an iso-osmotic nitrate solution. The rates of chloride efflux were calculated by linear regression analysis of the first 30 points taken at four seconds intervals while the change of fluorescence was still linear. As in other cell types (27, 29) both ES-D3 and electroporated ES-D3 cells exhibited a low chloride efflux under baseline conditions when chloride was replaced by nitrate. Stimulation of PKA by addition of FSK+IBMX significantly increased the CFTR-dependent chloride efflux D3 ES cells. Addition of the CFTR inhibitor, glibenclamide (100 μM) (30) to the perfusion solutions before and during the next FSK+IBMX stimulation inhibited this PKA-dependent increase to basal levels.

4. RESULTS

4.1. SDF nucleofection

A SDF (783-bp) was synthesized as previously described (2,31) introducing the 3-bp deletion (ΔF508) and a silent mutation, that gives rise to a unique KpnI restriction enzyme cleavage site (Figure 1A). The ΔF508SDFs were delivered into cultured ES cells (D3), using the AMAXA Nucleofection System. Preliminary experiments performed in D3 cells with green fluorescent protein plasmid (pEGFP) showed that the optimal electroporation program (A-30), gave a transfection efficiency of ~ 45-50% and survival of ~90% (data not shown).

4.2. DNA analysis

At 5 days after transfection, cells were harvested to assess whether SDF sequences were correctly incorporated into the genomic DNA. At this time point ~5-6 $x10^6$ cells were present, equivalent to ~8-10-fold population doublings. Given the original SDF dose there should now be a maximum of 3,200 SDF molecules per cell assuming that none have been degraded. Given this SDF dosage the potential of generating a PCR artifact is unlikely (32). In fact no PCR artifact was detectable if a quantity of $\leq 10^4$ (or $\leq 10^6$ depending on the primer) free SDF/cell is mixed within cells and the genomic DNA isolate is amplified.

Transfected cells were analyzed by AS-PCR, and subsequent KpnI restriction enzyme digestion of the PCR amplification products (Figure 1B). SFHR-mediated, site-specific deletion (ΔF508) was detected. KpnI digestion of the PCR amplicons from DNA of

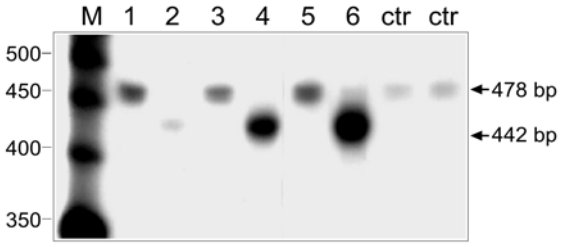

Figure 2. Polyacrylamide gel analysis of allele-specific PCR amplification products generated from the genomic DNA of transfected cells and digested with KpnI. Lane M: 50-bp DNA ladder (Invitrogen, Carlsbad, CA). Lanes 1-6: amplicons derived from ES cells transfected with different quantities of SDF; lane 1 and 2 correspond to cells transfected with 6.4 x 10^5 SDF/cell, lane 3 and 4 with 1.2 x 10^6 SDF/cell, and lane 5 and 6 with 1.9 x 10^6 SDF/cell. Lanes 1, 3 and 5 are amplicons obtained with primers mCF4/mCF3N while lanes 2, 4 and 6 with primers mCF4/mCF3ΔF. *Ctr* sample is derived from ES cells transfected with *Smn*-SDF (8). All samples amplified with primers mCF4/mCF3N and mCF4/mCF3ΔF and digested by KpnI. The 442-bp band is the result of KpnI digestion of SFHR-modified genomic DNA. Arrows indicate the molecular weight of amplicons and of its digestion products.

electroporated cells was also observed with the different doses of SDF, thus demonstrating SFHR-mediated site-specific modification (Figure 2). Specifically DNA sample amplified with the wild type and ΔF508-specific primers and then digested by KpnI. Only the mutant allele was digested, indicating that SFHR-mediated modification had occurred. To further substantiate the specificity of the molecular analysis, two other control analyses were carried out. First, different amounts of ΔF508-SDF (from 10^6 to 10^{-1} molecules per cell) were mixed with mouse genomic DNA of untransfected cells. Moreover mouse ES-D3 cells were transfected with SDF homologous to *Smn* gene and then extracted and analyzed (Figure 2). Both samples were used as templates to assay for any potential PCR-mediated artifacts that might arise from the amplification of the SDF. No anomalous PCR amplification products were observed (data not shown) .

4.3. Analysis of mRNA

To evaluate if SFHR-modified DNA was properly expressed, mRNA from transfected cells was converted to cDNA after DNAse treatment, and then amplified by allele specific-PCR (Figure 1C). Two amplicons, 237-bp and 234-bp, were generated for the wild type and ΔF508 CFTR alleles, respectively (Figure 3). These PCR products were subsequently cloned and sequenced to confirm the presence of both modifications (the deletion and the restriction site) within the SDF (TA Cloning, Invitrogen, San Diego, CA; http://www.invitrogen.com). Sequence analysis showed the presence of the expected ΔF508 mutation together with the KpnI restriction site, as indicated by arrows (Figure 3). Both variations were absent in wild-type allele. At the same time, clones were screened for the presence of the KpnI restriction site by PCR amplification and enzymatic digestion.

The ΔF508 transcript was quantified by TaqMan-based real-time quantitative RT-PCR, using the ABI PRISM 7700 Sequence Detection System (Applera; http://www.applera.com). Two oligonucleotide probes homologous to wild type and ΔF508 alleles respectively were designed. Control mRNA was isolated from ΔF508 homozygote, heterozygote, and *CFTR* knockout mouse cells and included in the analysis (data not shown). Real-time PCR analysis of each sample was performed in triplicate and the individual experiments were repeated at least three times. The "mutated allele", containing ΔF508, was expressed at about 12% (Figure 4). These results indicate that the SDF-modified allele was transcribed and expressed in D3 cells. Cells transfected with *Smn*-SDF and untransfected ones were negative for the expression of the ΔF508 allele.

4.4. CFTR activity in transfected and untransfected cells

To examine whether ΔF508/SDF electroporation into D3 ES cells was able to induce a variation in the CFTR-dependent chloride efflux, we performed spectrofluorimetric measurements in both treated and untreated cells. Figure 5 illustrates the experiments performed on D3 (A) and on electroporated D3 cell populations (B), seeded on glass coverslips and loaded with the chloride sensitive dye (MQAE). As shown in figure 5A (left panel) PKA stimulation by addition of FSK+IBMX increased chloride efflux in D3 cells. In fact this is clearly shown by the significant slope increase of the change in fluorescence (fig. 5, left panel) and the decline of the slope in addition of the specific CFTR inhibitor, glibenclamide before and during the following FSK+IBMX stimulation almost completely inhibited this increase. These data suggests that the PKA-dependent chloride efflux was mainly due to CFTR stimulation. Figure 5A (right panel) summarize the data from fourteen independent experiments. In the histogram, the empty bar represents CFTR-dependent chloride efflux calculated as the difference in alterations of FSK+IBMX stimulated fluorescence in the absence (light gray bar) and presence (dark bar) of glibenclamide. In contrast, in transfected ES cell population (Figure 5B), FSK+IBMX treatment induced only a weak increase of the CFTR dependent efflux, which was slightly inhibited by glibenclamide addition. These data suggest that CFTR activity was decreased in electroporated D3 cells, with respect to the untransfected control. Comparing these results, it can be seen that CFTR-dependent chloride efflux was 58% lower in electroporated cells respect to the untreated ones (0.009 ± 0.002, n=13 glibenclamide sensitive Cl^- efflux ($\Delta(F/F_0)$/min) in transfected ES cells vs 0.022 ± 0.002, n=14, in ES untreated cells, respectively). It is also important to note that the inhibition of CFTR activity revealed in transfected cells was exclusively SDF-mediated, since cells electroporated with fragments homologous to *Smn* locus behaved as untreated ES cells (0.025 ± 0.005, n=4 glibenclamide sensitive Cl^- efflux ($\Delta(F/F_0)$/min).

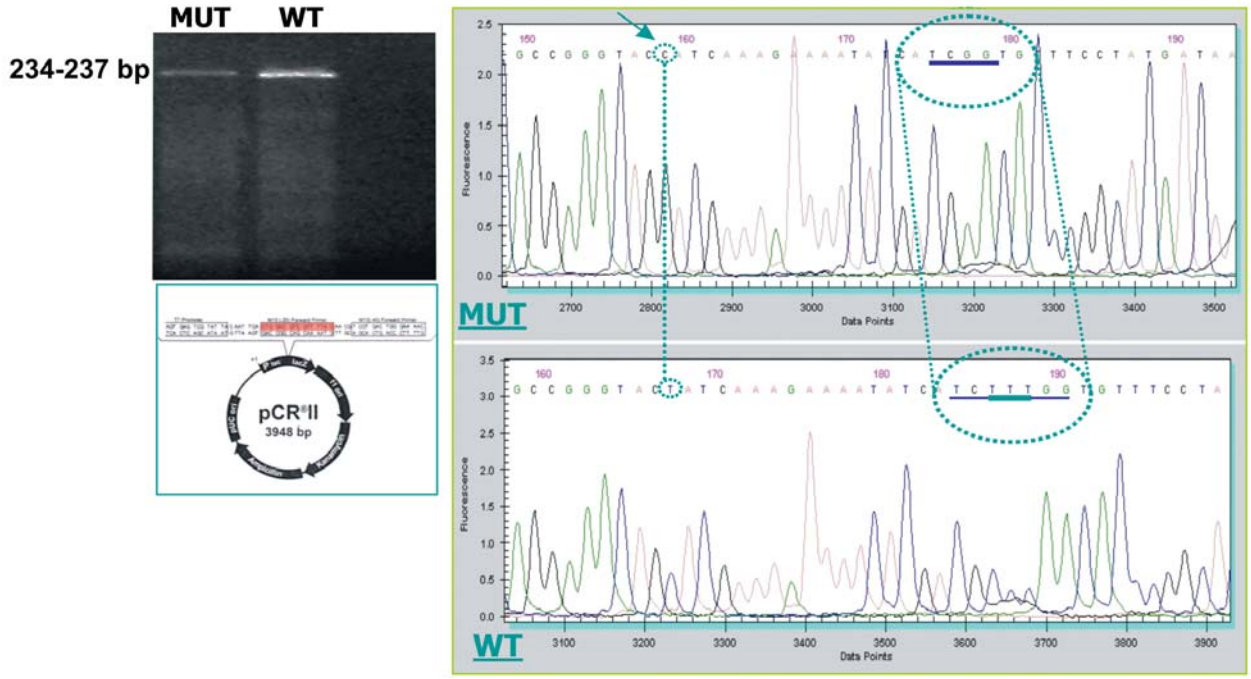

Figure 3. Gel electrophoresis analysis of AS-PCR performed on mRNA-derived cDNA from transfected ES cells. Wild type and ΔF508 amplicons were generated using primers mCF11/mCF3N and mCF11/mCF3ΔF, respectively (see Figure 1 C). These amplicons were cloned into vectors which were then isolated clones and sequenced. Sequencing of mCF11/mCF3ΔF (MUT) exclusively showed the presence of both the ΔF508 allele and the KpnI restriction site (arrows) indicating that the SDF-derived sequences were expressed as CFTR mRNA.

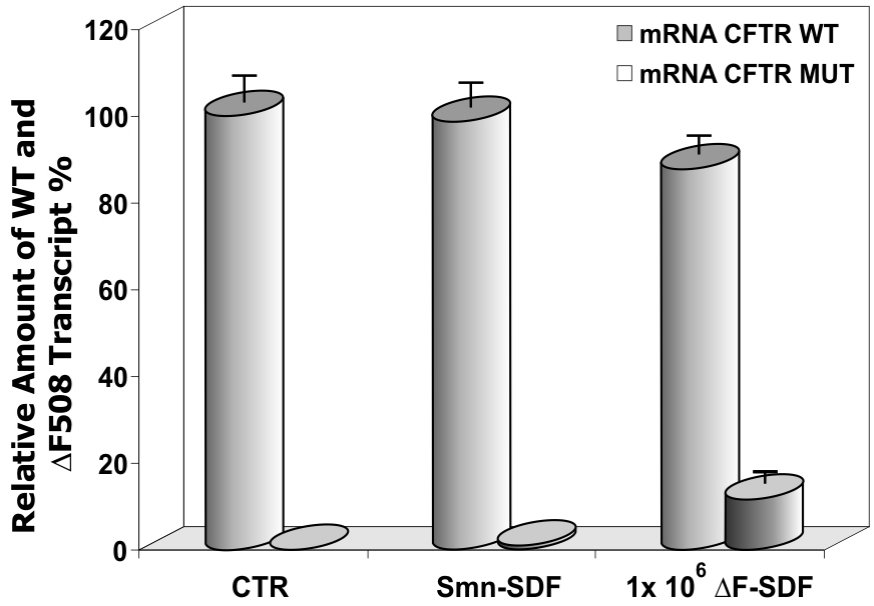

Figure 4. Quantitative PCR analysis of the ΔF508 and wild type *Cftr* transcript in transfected D3 ES cells. Open columns represent the wild type transcript, while shaded columns represent the ΔF508 transcript. Sample 1 corresponds to cells transfected with no SDF, sample 2 to cells transfected with SDF homologous to *Smn* gene, and samples 3 to cells transfected with 1 x 10^6 ΔF508-SDF/cell. The values obtained from treated cells represent the mean of at least three independent experiments that were performed in triplicate. Values were significantly different from those obtained with untreated cells. Error bars indicate the SD. A p value of < 0.05 was considered statistically significant.

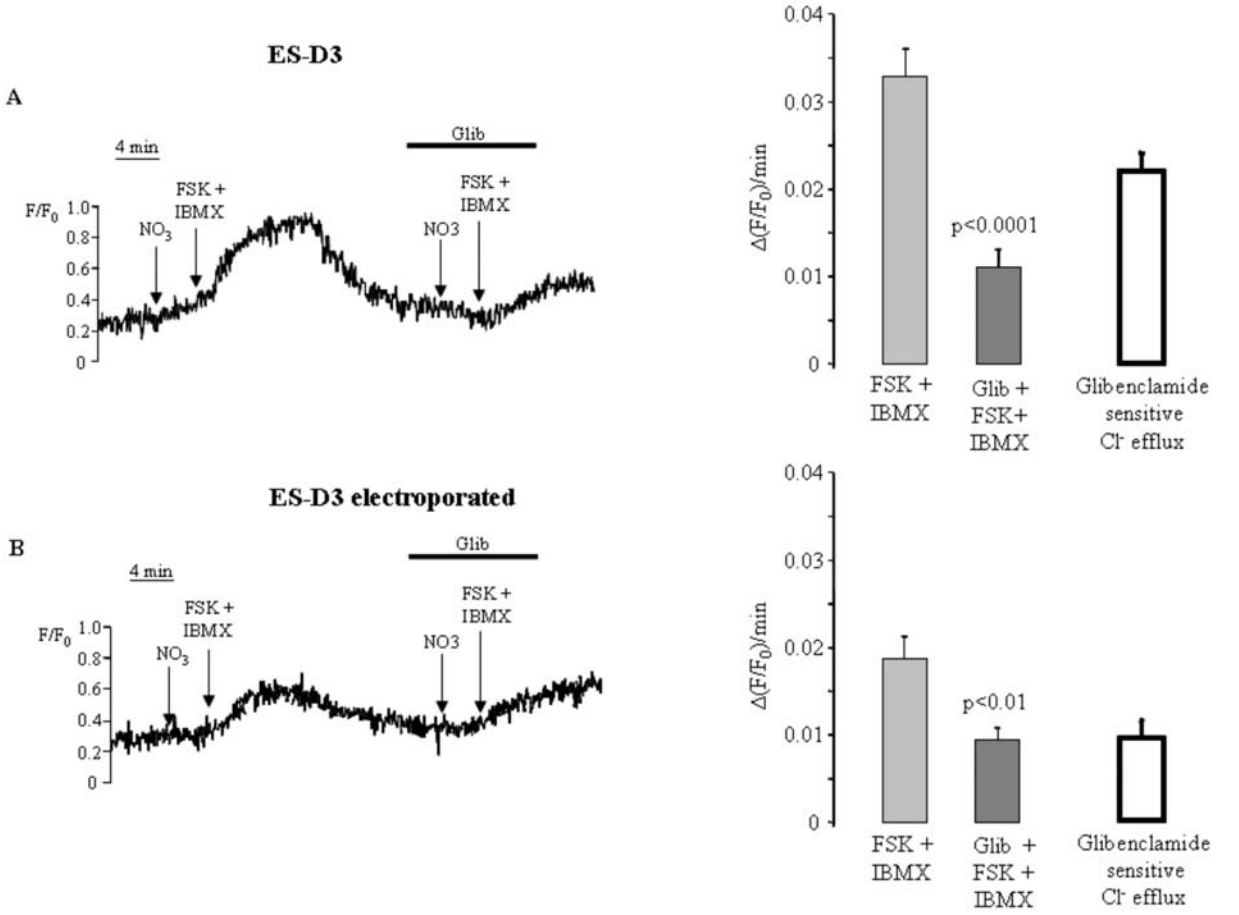

Figure 5. CFTR-dependent chloride efflux of ES-D3 (A) and electroporated ES-D3 (B) cell populations. Typical recordings (left panels) obtained by spectrofluorimetric analysis of the entire ES-D3 and electroporated ES-D3 populations seeded on glass coverslips (see Methods) show the changes in intracellular Cl--dependent MQAE fluorescence (expressed as the F/F0 ratio) when the cells were treated with 10 μM FSK plus 500 μM IBMX following substitution of chloride by nitrate in the absence or presence of 100 μM glibenclamide. Glibenclamide was applied before the next FSK+IBMX stimulation and remained during the entire chloride efflux measurement. The right panels show the summary of data from n=14 and n=13 experiments for ES-D3 (A) and electroporatedd (B) ES-D3 cells respectively. The glibenclamide-sensitive, CFTR-mediated chloride efflux rates (empty bars) were calculated as the difference in the F/F0 ratio per minute ((F/F0)/min) in the absence of (light gray bar) and presence of (dark bar) glibenclamide. Each bar represents the mean ± S.E. The data were compared by using the two-tailed, paired Student's t test analysis. $p < 0.05$ was considered statistically significant.

The spectrofluorimetric measurements were performed on a total population of electroporated WT ES cells. To further investigate whether the ES cells were homogenously modified by SFHR, we analyzed and compared CFTR-dependent chloride efflux between "single" colonies of ES treated and untreated cells by a video imaging technique. To do this we analyzed an average of 4-6 regions for each colony (each one containing 20-25 cells) by video-imaging to verify the cell homogeneity of each colony.

In Table 1 we have summarized all the experiments performed on both D3 electroporated and not electroporated cell colonies. From our results appears evident that transfected colonies were all homogenous because all regions examined wihin the same clone showed a similar significant CFTR dependent chloride efflux (0.035 ± 0.003 Δ(F/F0)/min n=35 regions analyzed in seven ES-D3 colonies). Moreover on twelve electroporated ES colonies analysed by us, eight were successfully mutated because their CFTR–dependent chloride efflux was not significantly different from zero (0.002 ± 0.002 Δ(F/F0)/min; 33 regions examined). The remaining four colonies showed a CFTR-dependent chloride efflux that was not significantly different from the untransfected ones revealing an unsuccessfully SDF-modification (0.037 ± 0.004 Δ(F/F0)/min; 16 regions examined). This confirmed again the heterogeneity of the transfected cell population.

Table 1. CFTR-dependent chloride efflux in ES-D3 and ES-D3 electroporated cells measured by video imaging

	Number of colonies	Regions examined	CFTR dependent chloride efflux $\Delta(F/F_0)$/min
ES-D3	7	35	0.035 ± 0.003
ES-D3	8	33	0.002 ± 0.002
electr	4	16	0.037 ± 0.004

Values are mean ± S.E. ES-D3 cells form colonies of 23-25 cells after four days from the seeding on glass coverslips coated with 0.1% gelatin. The CFTR-dependent chloride efflux was detected by simultaneous fluorescence measurements from different 4 to 6 regions of individual colonies.

5. DISCUSSION

Gene targeting by homologous replacement makes it possible to precisely manipulate genomic DNA and maintains genetic integrity by retaining the relationship between the protein coding sequences and the gene-regulatory elements (5). This aspect of homologous replacement overcomes any potential for inappropriate gene expression either in the amount of protein produced or in the type of cell expressing the gene (33). A recent study suggests that preclinical experimental treatments involving transgenes should include long-term follow-up before they enter clinical trials (34). Authors reports a long latency period before lymphomas develop in mice transplanted with cells that have been transduced with LV-IL2RG. This observation further highlights the need to develop vectors capable of regulated therapeutic gene expression.

Oligonucleotide-mediated modification has been applied by a number of different groups both *in vitro* and *in vivo* to modify both plasmid and genomic DNA targets (35-42). Among the various oligonucleotide-based gene targeting approaches, SFHR has been shown to correct specific mutations at a target locus (5). In a recent study SFHR was shown to restore the *SMN* full length protein in human SMA cells obtained from chorionic villi, demonstrating the feasibility of using this approach to stably correct human fetal cells (8). Another study described genotypic and functional correction of a point mutation in the gene encoding the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (21). In addition, a number of studies have shown specific modification of the CFTR gene by SFHR (2,4,6,8,17,19,43).

Based on these studies, the potential of SFHR-mediated modification for "*in vivo*" or "*ex vivo*" gene therapy of monogenic disorders is significant when compared to the cDNA-based "gene complementation" approaches (5, 44-49).

This study showed that it was possible to insert a 3-bp (ΔF508) deletion into the genomic DNA *Cftr* gene of mouse embryonic stem cells by SFHR following electroporation (nucleofection) of a 783-bp ΔF508 fragment containing the unique KpnI restriction site. As a result, the SDF-derived ΔF508 mutant mRNA was expressed.

Furthermore potential PCR artefacts that could result from the presence of free SDF within the cell (20, 28, 50, 51) was not detected. To minimize the potential for artifact, the PCR primers used were outside the region of homology defined by the SDF. In addition, the SDF copy number at the time of analysis was about 3200 molecules per cell, assuming that there was no degradation or loss of the transfected SDF. This number is less than that required to give rise to any PCR artifacts as already reported in DNA mixing reconstitution analyses (20, 28). Moreover, the treatment of the isolated RNA with DNase eliminates any contaminating SDF that might be present in the crude RNA isolate. Consistently with the molecular analysis, CFTR channel activity was significantly reducted in transfected ES cells. Using spectrofluorimetric measurements of the entire population of the cells, we specifically found that CFTR-dependent chloride secretion was 58% lower in ES electroporated cells with respect to controls. Video-imaging measurements performed on single ES clone, demonstrated in the same time that each clone is composed by homogeneous cells but not all clones underwent to SFHR-mediated modification. In fact different regions within the same clone exhibited the same CFTR-dependent chloride efflux, but only 8 of 12 examined clones showed a complete inhibition of CFTR-dependent chloride efflux.

As far as we know, the present study applies for the first time a functional test for evaluating the specific SFHR-induced modification in ES cells, avoiding any artefacts due to the presence of the free SDF, not integrated within genomic DNA, as recently reported (45).

In addition to its role as a tool for developing an *in vitro* means for understanding the pathophysiology of monogenic disorders, SFHR can be applied to ES cells for therapeutically correcting genetic mutations and repairing disease dependent tissue damage (5). SFHR has already been used for modifying hematopoietic stem cells (5,20-23) that have been shown to have the capacity to differentiate into human airway epithelial cells (52). Mouse ES cells have also been shown to generate a fully differentiate and functional tracheobronchial airway epithelium (53-55) and could also potentially be applied to repair damaged CF airways.

Moreover, mutating genes in ES cells by homologous recombination has been a powerful research tool for developing animal models of human disease. The approach described here could potentially augment these classical homologous recombination strategies in mice to develop a range of animal models through nuclear transfer (5, 20, 24, 56).

In conclusion, the present study represents the basis for developing innovative cell and gene-based therapeutic strategies for CF or other monogenic disease. While it has not yet been possible to effectively carry out somatic cell nuclear transfer in human oocytes, the

potential of generating patient derived stem cells with corrected mutant genes could conceivably translate into a significant improvement and possible cures for many inherited diseases.

6. ACKNOWLEDGMENTS

Federica Sangiuolo, Maria Lucia Scaldaferri contributed equally to this work. This study was supported by grants from the Italian Ministry of Health, Italian Ministry of University and Scientific Research (MIUR) and by FFC Italian Foundation (grant: FFC 5# 2005) and partially by Medusa Film Roma. D.C. Gruenert is supported by NIH Grants DK066403 and HL80814 and grants from the Cystic Fibrosis Foundation and Pennsylvania Cystic Fibrosis, Inc.

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Abbreviations: CFTR: cystic fibrosis transmembrane conductance regulator, CF: cystic fibrosis, SDF: small DNA fragment, *Smn*: survival motor neuron gene , HR: homologous recombination, SFHR: small fragment homologous replacement, ES cells: embryonic stem cells

Key Words: Homologous Replacement, Real-Time PCR, SFHR, Embryonic Stem Cells, CFTR

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