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RESEARCH ARTICLE

Oligonucleotide-directed single-base DNA alterations in mouse embryonic stem cells

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We have investigated the use of single-stranded oligodeoxynucleotides (ssODN) to produce specific single-base alterations in episomal and chromosomal DNA in mouse embryonic stem (ES) cells. Two different reporter genes, EGFP *and* LacZ*, each with a single point mutation that inactivates reporter activity, were used. ssODN homologous to the target sequence, except for a single mismatch at the mutant base, were used to correct the mutant reporter genes. When tested in CHO-K1 cells, the ssODN showed correction rates of 0.5–1.0%, consistent with prior reports. ssODN in the antisense orientation provided higher rates of gene conversion than those in the sense orientation for both reporter genes. Nuclear extracts from mouse ES cells exhibited nearly the same correction activity as extracts from CHO-K1 cells. ssODN corrected the mutant bases of both episomal and chromosomal mutant reporter genes in mouse ES cells. Although the efficiency of gene correction observed in ES cells is low, approximately 10^{-4}, these results demonstrate that ssODN can produce single-base alterations in the genomic DNA of mouse ES cells. As conversion efficiency is improved by the continued development of oligonucleotide structure and DNA delivery methods, ssODN could be used to produce ES cells with specific mutations in any gene in a single step. The targeted ES cells could in turn be used to create accurate mouse models of inherited diseases.*
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Introduction

Gene targeting techniques have created a revolution in biology, allowing for production of mice with targeted disruption of specific genes. Mice generated by these techniques have become invaluable tools to study the function of proteins *in vivo*.[1,2] Current gene targeting techniques use homologous recombination in mouse embryonic stem (ES) cells to introduce site-specific modifications into the mouse genome. Using variations on this fundamental approach, it has become possible to produce mice with genetic alterations ranging from large deletions, to simple disruptions, to more subtle changes such as point mutations.[2,3] As a testament to the power of these techniques, thousands of mice with disrupted genes have been generated since the technique was introduced.[1,4]

While gene targeting experiments are often successful, there are several limitations to the technique. Not all knockout animals demonstrate the predicted phenotype. It has been hypothesized that this is due to compensation for the missing protein by redundancy in protein function.[5] Many genes have critical functions in development, and their deletion can lead to embryonic or early postnatal lethality, preventing analysis of gene function in adult animals.[6] Deletion of large portions of targeted genes may also lead to unintended loss of regulatory elements governing the expression of other genes, which in turn can lead to unexpected phenotypic variation.[7] Finally, while recessive disease is often caused by insufficient protein expression, and thus can be modeled effectively by knockout mice, dominant diseases are most often caused by dominant negative or gain of function mutations; knockout mice are thus not always accurate models of dominant disease. An alternative approach for producing mice with specific mutations would be useful.

One clear example of the differences between mice with knocked-out genes and mice with a specific mutation is mice with disruptions of the fibroblast growth factor receptor 3 (*FGFR*3) gene. Achondroplasia, the most common form of dwarfism, is caused by a missense mutation (G380R) in *FGFR3*, and is associated with premature termination of bone growth. *FGFR3*-deficient mice, produced by knockout techniques, exhibit bone overgrowth. In contrast, mice with the G380R mutation, which was introduced by conditional gene targeting or 'knock-in' techniques, have a dominant dwarf phenotype with early cessation of bone growth.[8]

As indicated by the example cited above, conditional gene targeting has extended the usefulness of gene manipulation techniques to allow for the creation of subtle alterations in genomic DNA, including single-base changes.[9] While conditional gene targeting is a powerful

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technique to produce animal models of disease, these techniques can be difficult to use. First, multiple manipulations of mouse ES cells are required to produce mice with subtle changes in their DNA. With each round of transfection and selection, the ability of the targeted ES cells to contribute to the germline is diminished. An alternative to multiple alterations of DNA in ES cells is to produce mice with targeted conditional alleles and then cross them with mice that express the desired recombinase in the appropriate tissue. While diminishing the manipulation of ES cells, this requires the additional time associated with the breeding steps. Finally, there is evidence that expression of Cre recombinase in mammalian cells can be genotoxic.[10] Alternative methods to introduce targeted mutations into endogenous genes in a single step would be very useful.

A potential alternative to traditional gene targeting strategies is to use oligonucleotides to modify the genomic DNA of mouse ES cells. Oligonucleotides have been used to introduce alterations into cellular DNA for many years. For example, oligonucleotides ranging in size from 20 to 70 bases have been used successfully for site-directed mutagenesis *in vivo* in yeast and mammalian cells.[11–13] Triplex forming oligonucleotides coupled to functional chemical groups or double-stranded DNA have been used to alter, specifically or non-specifically, genomic DNA in cultured cells and *in vivo*.[14–18]

In 1996, Yoon *et al*[19] demonstrated that chimeric RNA–DNA oligonucleotides could introduce single-base alterations into episomal DNA, by what was called chimeroplasty. Chimeroplasty was subsequently used to introduce single-nucleotide conversions into the genomic DNA in cultured lymphoblasts, hepatoma cells and melanocytes.[20–22] Successful use of chimeroplasty to introduce single-nucleotide conversions in liver, skin and muscle cells *in vivo* has also been reported.[23–26]

Attempts to use chimeroplasty for gene correction experiments have not always been successful. This may be due in part to difficulty in synthesizing and purifying double-stranded chimeric oligonucleotides. In addition, different cell types exhibit variation in the frequency of gene targeting events, perhaps due to different levels of the enzymes required for chimeroplasty.[27,28] Most recently, two groups have determined that single-stranded oligodeoxynucleotides (ssODN), protected from degradation by phosphorothioate linkages or 2′-O-methyl RNA groups at both ends, can produce single-base pair changes in DNA.[29–31] These ssODN are easier to synthesize and purify than the original double-stranded chimeric RNA–DNA oligonucleotides, and produce gene conversion at similar frequencies to that reported for double-stranded molecules.[30]

The evidence available to date suggests that oligonucleotide-directed gene conversion requires mismatch repair machinery to operate. This has been confirmed in cell-free systems.[32] Additional evidence suggests that homologous recombination may also be required.[28] Mouse ES cells are thus an attractive system in which to use oligonucleotides to produce subtle alterations in DNA, as they have active homologous recombination and mismatch repair systems.[33,34] In addition, mice produced from ES cells with specific single-base changes would be excellent models of genetic diseases. To date, there are no reports of the use of oligonucleotide-directed DNA alteration in mouse ES cells.

We have tested the feasibility of using ssODN to introduce single-base changes into reporter genes in mouse ES cells. Our results show, using two different reporter systems, that ssODN can be used to produce specific single-base alterations in the genomic DNA of mouse ES cells.

Results

EGFP and β-galactosidase reporter systems and oligonucleotides

To provide simple assays for detecting single-base changes produced by synthetic oligonucleotides, we chose to use two reporter gene systems. First, we developed a reporter system based on the enhanced version of green fluorescent protein (EGFP). EGFP autofluoresces, thus providing an easily detectable marker, and is well tolerated by mammalian cells.[35,36] We introduced several missense and non-sense mutations into the *EGFP* gene to turn off protein fluorescence. These mutant EGFPs were then cloned into the eukaryotic expression plasmid pcDNA3.1 (Invitrogen), and transfected into Chinese Hamster Ovary (CHO)-K1 cells. Mutation G67R (nucleotide change G to C at base 202 in *EGFP*) in the EGFP chromophore and the non-sense mutation Q177X (nucleotide change C to T at base 532 in *EGFP*) produced no detectable fluorescence, as determined by both microscopy and fluorescence activated cell sorting (FACS) analysis (see Figure 2), and were chosen for further use.

We next designed oligonucleotides to correct the G67R and Q177X mutations in EGFP; successful use of the oligos would thus produce fluorescent EGFP. The oligonucleotides we initially produced were of the original double-stranded design.[19] Although we found these to be active, we converted to using ssODN as they are more active, and easier to make and purify.[29,30] ssODN in both sense and anti-sense orientations were evaluated. Both 2′-O-methyl groups and phosphorothioate linkages at the 3′ and 5′ ends were used for nuclease protection.[29,30] Structures of the oligonucleotides used in these experiments are shown in Figure 1.

The second reporter system, which has been used previously, is a *LacZ* mutant with an E523K mutation (G to A at nucleotide 1651 of plasmid pCH110).[28] We also prepared oligos to correct the E523K mutation in *LacZ* (Figure 1C). Again, successful use of these ssODN would correct the E523K mutation, and produce enzymatically active β-galactosidase.

Gene conversion in CHO cells

To determine if our oligonucleotides were active, we transiently transfected CHO-K1 cells with plasmids containing mutant *EGFP* or *LacZ* genes, followed by a correcting oligonucleotide. Cells transfected with plasmid alone, or with plasmid plus control oligonucleotides without a mismatch (eg EGFP G4), were used in separate culture wells as controls. Two days after transfection, the cells were either stained with X-gal to detect β-galactosidase activity or analyzed by fluorescence microscopy and FACS to detect EGFP. Results from a representative EGFP experiment are shown in Figure 2. A summary of results from these experiments is presented in Table 1.

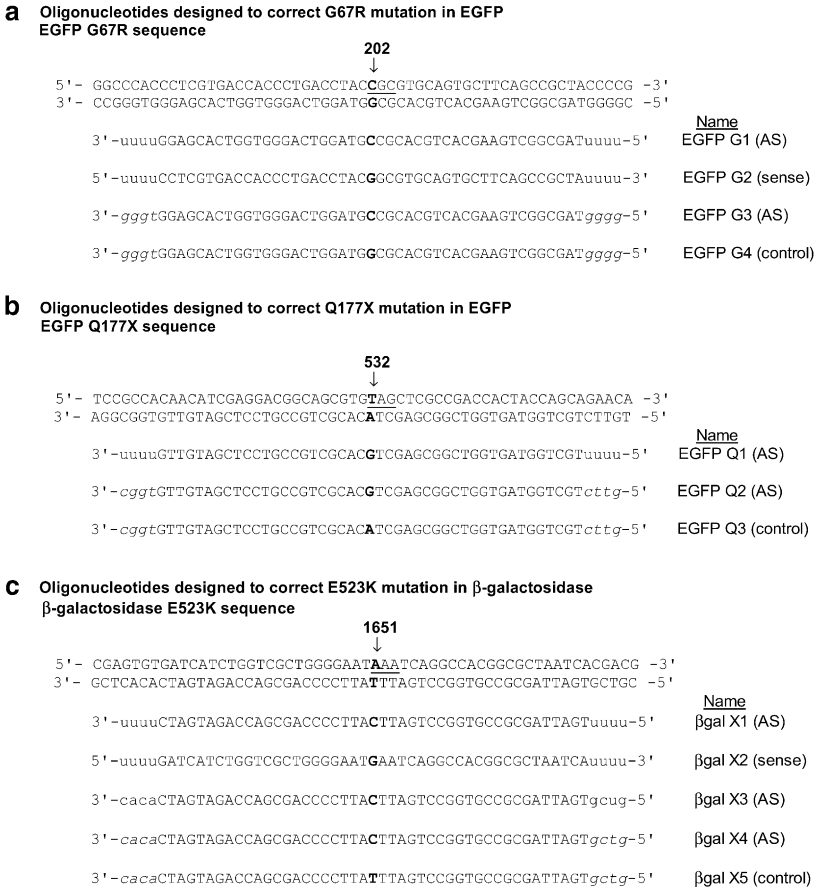

Figure 1 *Oligonucleotide sequences. Oligonucleotides used with the different mutant reporter genes are indicated. For each reporter gene, the mutant DNA sequence is shown, and the mutant bases indicated in bold. The mutant codon for each reporter is underlined. Lowercase letters indicate 2′-O-methyl RNA and uppercase indicates DNA. Italicized lowercase letters indicate phosphorothioate linked DNA. AS=antisense.*

As can be seen in Figure 2, control transfections using the G67R mutant version of EGFP with control ssODN *EGFP* G4 demonstrated no fluorescent cells (panels a–c). In contrast, when correcting oligonucleotides were used, fluorescent cells were detected in the culture plate (panels e, h) and by FACS analysis (panels f, i). Comparison of the M2 regions of the FACS histograms, indicating cells with fluorescent signal above background, showed 0.57% of cells were fluorescent following treatment with the EGFP G1 oligonucleotide (panel f), and 0.86% fluorescent cells following treatment with oligonucleotide EGFP G3 (panel i). This frequency of correction is consistent with prior reports.[30] These results have been reproduced multiple times, with several different preparations of plasmid and oligonucleotides. Similar results were also obtained using the Q177X mutant of EGFP and appropriate oligonucleotides (data not shown).

Table 1 Oligonucleotide-directed gene conversion in CHO cells[a]

Oligonucleotide	*Expt 1*	*Expt 2*	*Expt 3*	*Mean ± SD*
LacZ conversion – pCH110-g1651a plasmid				
βgal X1 (AS)	0.61	0.25	0.55	0.47 ± 0.19
βgal X2 (S)	0.23	0.22	0.37	0.27 ± 0.08
βgal X3 (AS)	0.74	0.42	0.52	0.56 ± 0.16
βgal X4 (AS)	1.02	0.87	2.78	1.56 ± 1.06
βgal X5 (control)	0	0	0	0
EGFP conversion – pcDNA3-G67R-EGFP plasmid				
EGFP G1 (AS)	0.57	0.45	0.29	0.44 ± 0.14
EGFP G2 (S)	0.01	0.02	0.02	0.020 ± 0.006
EGFP G3 (AS)	0.86	0.51	0.47	0.61 ± 0.21
EGFP G4 (control)	0	0	0	0

[a]For LacZ experiments, data are presented as the percent gene conversion (number of blue cells/well divided by the number of cells transfected (5×10^4) × 100). For EGFP experiments, data are the percent fluorescent cells determined by FACS; 50 000 cells were evaluated for each data point.

Similar results were also obtained using the mutant *LacZ* reporter plasmid and correcting ssODN. The data from the β-galactosidase reporter experiments in CHO-K1 cells are shown in Table 1. Although there is considerable variation between repetitive experiments, the relative order of ssODN activity is consistent across the three experiments. As determined previously, antisense oligonucleotides appear to be the most active in stimulating gene conversion. Phosphorothioate-protected DNA oligonucleotides reproducibly generated the highest rates of gene conversion (βgal X4), even when compared to 2′-O-methyl RNA protected oligonucleotides with the same number of homologous bases (βgal X3), although these differences were not statistically significant. These results confirm the activity of the ssODN using two different reporter gene systems.

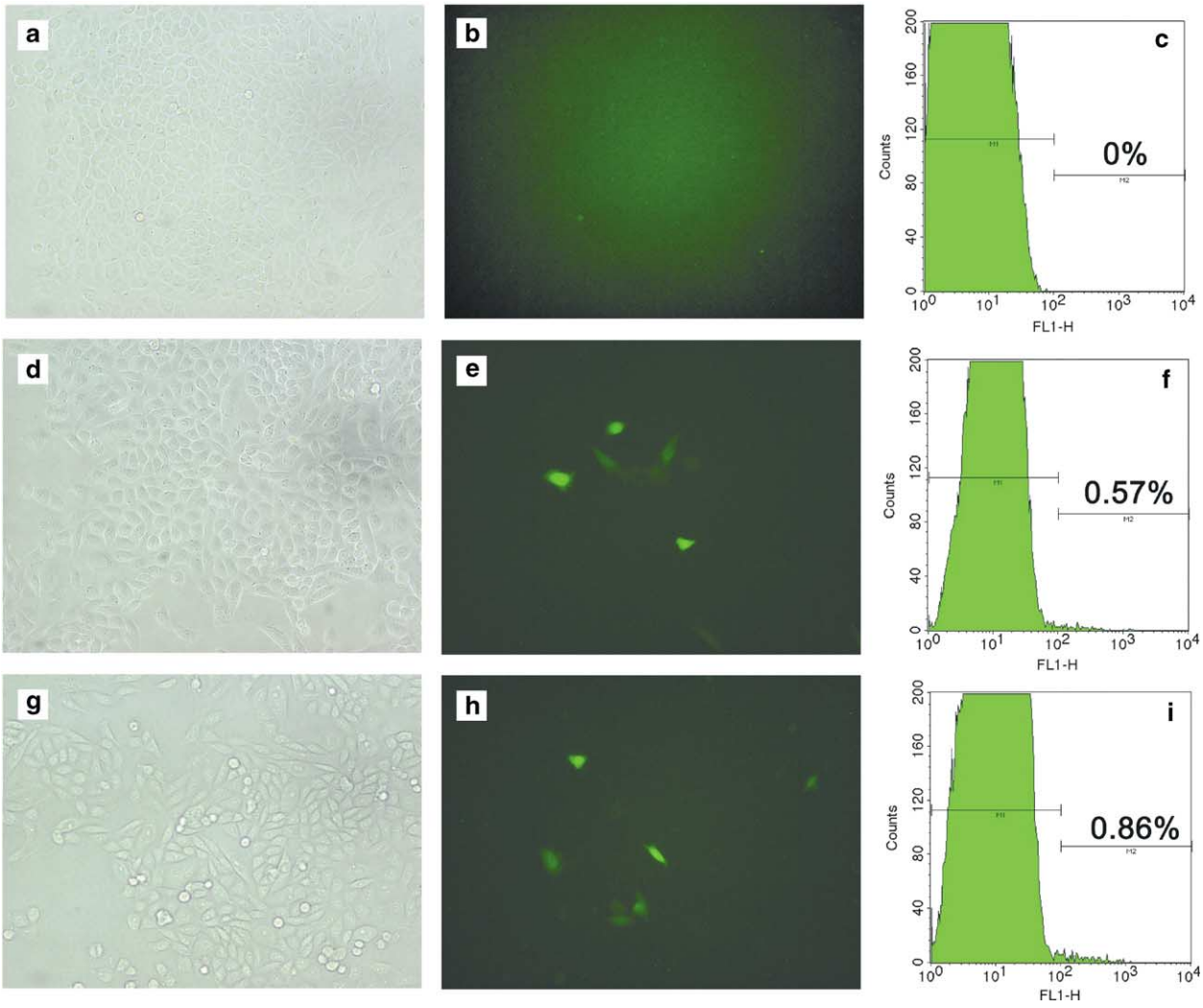

Figure 2 *Episomal gene conversion in CHO-K1 cells. CHO-K1 cells were transfected with the G67R mutant* EGFP *plasmid followed by control (a–c) or correcting oligonucleotides (d–i). Cells were viewed by phase (a, d, g) and fluorescence (b, e, h) microscopy, and then analyzed FACS (c, f, i). As can be seen, control oligonucleotide EGFP G4 did not produce any glowing CHO-K1 cells (b, c). Transfection with correcting oligonucleotide EGFP G1 (d–f), corrected the mutant* EGFP *plasmid in 0.57% of cells (e, f). Transfection with correcting oligonucleotide EGFP G3 (g–i) corrected the mutant* EGFP *plasmid in 0.86% of cells (h, i).*

In vitro reaction by ES cell extract

It has been reported that the ability of different cell types to carry out oligonucleotide-directed gene conversion varies.[27,28] This variability is thought to be due to the presence or absence of the enzymes needed to perform homologous recombination and/or mismatch repair. In order to determine if ES cells have the enzymatic machinery needed to carry out gene conversion, we tested nuclear extracts of mouse ES cells using an *in vitro* method.[28] Mutant *LacZ* reporter plasmid and correcting or control ssODN were incubated in nuclear extracts from several different cell types. Following this incubation, the plasmid DNA was extracted and electroported into P90C bacteria, which lack the entire *lac* operon. The resulting number of blue colonies was recorded. As can be seen in Table 2, mouse ES cell extract is at least as active as CHO-K1 extract at correcting the single-base mutation in the reporter plasmid. In contrast, embryonic fibroblast feeder cells, on which ES cells are cultured, showed less activity. These data indicate that mouse ES cells express the enzymes necessary for oligonucleotide-directed gene conversion.

Single-base conversions in ES cells – transient transfections

To test the ability of ssODN to produce single-base alterations in ES cells, we transiently transfected ES cells

Table 2 *In vitro* assay of gene conversion by nuclear extracts[a]

Cell type	*Expt 1*	*Expt 2*	*Man ± SD*
CHO cells	0.020 (20)	0.025 (25)	0.023 ± 0.004
Mouse ES cells	0.011 (11)	0.042 (42)	0.027 ± 0.022
Feeder cells	0.004 (4)	0.020 (20)	0.012 ± 0.011

[a]Data are presented as the percent blue colonies observed, with the actual number of blue colonies counted per 10^5 total colonies shown in parenthesis.

with either *EGFP* or *LacZ* reporter plasmid, combined with oligonucleotide. We have found that use of the cationic peptide nuclear localization signal M9 (CM9) in lipid-based transfections greatly enhances gene expression and improves oligonucleotide uptake in ES cells. Mouse ES cells lipofected with CM9 retain their pluripotency and contribute to the germline (Ma *et al*, submitted). We therefore used CM9 peptide for our ES cell transfections.

As can be seen in Figure 3, correcting oligonucleotide EGFP Q2 produced active EGFP in individual ES cells (panels c–f). No fluorescent ES cells were seen following transfection with plasmid alone, or with plasmid plus control oligonucleotide EGFP Q3 (panels a, b). Similar results were obtained with the G67R mutant *EGFP* reporter plasmid and appropriate ssODN (data not shown). As shown in Figure 4, βgal X4 oligonucleotide produced active β-galactosidase in ES cells, and detected

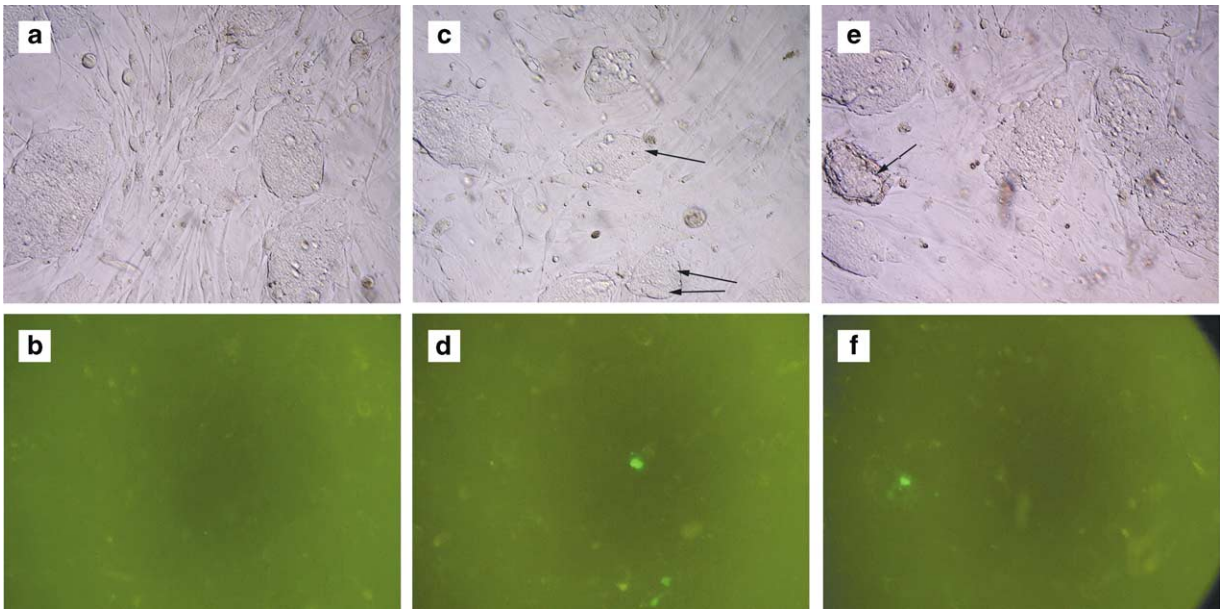

Figure 3 *Episomal gene conversion in ES cells – EGFP. ES cells were transfected with the Q177X mutant* EGFP *plasmid combined with control or correcting oligonucleotides. Cells were viewed with phase (a, c, e) and fluorescence (b, d, f) microscopy. Fluorescent ES cells (arrows) were detected following transfection with oligonucleotide EGFP Q2 (d, f), but not following transfection with control oligonucleotide EGFP Q3 (b).*

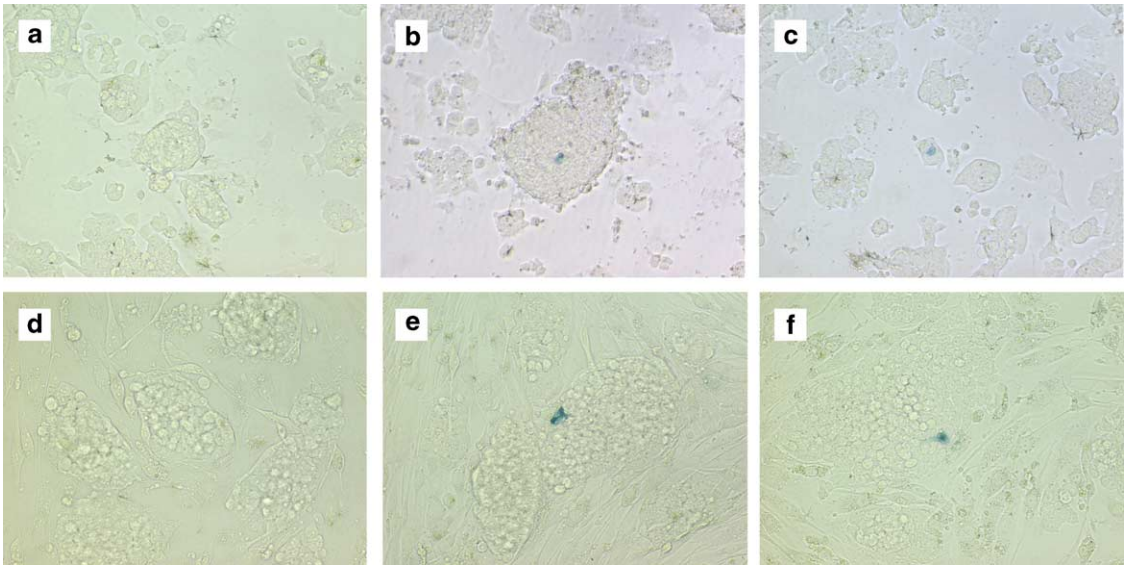

Figure 4 *Episomal gene conversion in ES cells – β-galactosidase. ES cells were transfected with mutant* LacZ *plasmid combined with the control oligonucleotide βgal X5 (a, d) or correcting oligonucleotide βgal X4 (b,c,e,f). Cells were stained with X-gal to detect β-galactosidase activity 48 h after transfection. Blue ES cells were detected following transfection with the correcting oligonucleotide, indicating correction of the mutant* LacZ *gene in ES cells. No blue cells were seen following transfection with control oligonucleotide.*

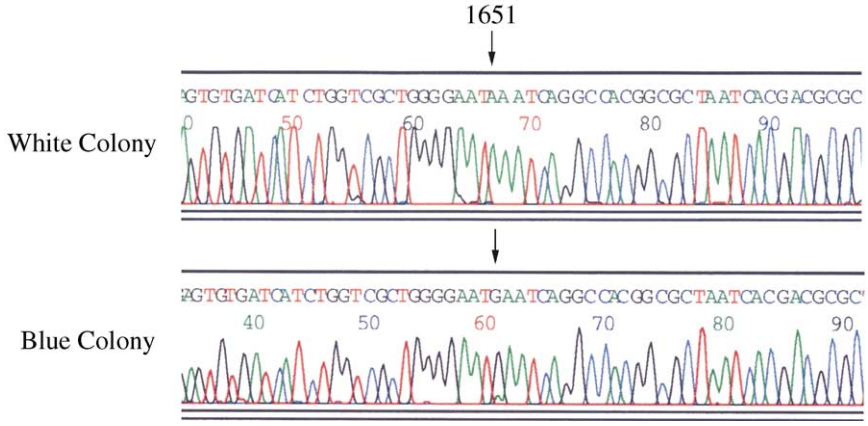

Figure 5 *Confirmation of specific gene conversion in ES cells. ES cells were transfected with mutant* LacZ *plasmid combined with control or correcting oligonucleotides. Cells were harvested 48 h after transfection, and Hirt DNA isolated. Hirt DNA was used to transform P90C cells. Blue colonies were observed only in DNA from transfections that included the correcting oligonucleotide βgal X4. Plasmid DNA isolated from these blue colonies demonstrates the specific A to G sequence correction at base 1651 of plasmid pCH110 (blue colony). In constrast, plasmid DNA isolated from white colonies from the control transfraction contains the mutant base A at position 1651 (white colony). No other sequence alterations were detected in the β-galactosidase coding regions of the isolated plasmids.*

by X-gal staining (panels b, c, e, f). To verify that the blue cells detected were ES cells, and not feeder cells, transfected ES cells were plated without feeders and then stained (Figure 4, panels a–c). Blue ES cells are clearly detected even in the absence of feeder cells. Correction of the single-base mutation in the *LacZ* reporter plasmid in ES cells was detected repeatedly in five separate experiments, with 10–30 individual blue ES cells/well of the six-well plate. ES cells treated with control oligonucleotide βgal X5 showed no β-galactosidase activity (panels a, d).

To verify that the blue cells observed following treatment with correcting oligonucleotide indicate β-galactosidase activity produced by specific correction of the G1651A mutation in the reporter plasmid, Hirt DNA was isolated from ES cells 48 h after transfection with plasmid and oligonucleotide βgal X4. The Hirt DNA was then used to transform P90C bacteria. The tranformed bacteria were plated on agar containing X-gal, and the resulting number of blue colonies was counted. Hirt DNA isolated from ES cells transfected with correcting oligonucleotide βgal X4 produced approximately seven blue colonies/10^5 total colonies. In contrast, Hirt DNA isolated from ES cells treated with control oligonucleotide βgal X5 produced no blue colonies. Plasmid DNA was isolated from five blue colonies from the βgal X4 treated ES cell DNA. Dye terminator sequencing of the entire β-galactosidase coding region in the plasmid DNA revealed specific base correction from A to G at position 1651 in the blue colonies, with no other base alterations noted (Figure 5). Plasmid DNA was also isolated from five white colonies grown from the control-treated ES cell DNA; in all cases, the mutant β-galactosidase coding sequence was detected, without any other alterations (Figure 5).

To verify that oligonucleotide-directed gene conversion can be used in multiple lines of ES cells, we tested two additional ES cell lines for this activity by transient transfection. TL1 and R1 mouse ES cells showed levels of gene-correction activity similar to that observed for AB2.2 ES cells (data not shown).

Single-base conversions of integrated reporter gene ES cells

To determine if ssODN can produce single-base alterations in the genomic DNA of mouse ES cells, we generated ES cells with an integrated copy of the mutant *LacZ* gene. As shown in Figure 6A, ES cell lines A12 and B13 have one copy of the mutant reporter gene integrated into their genomic DNA. These ES cell lines also produce the mutant β-galactosidase protein, as indicated by Western blotting (Figure 6b).

Transfection of ES cell lines A12 and B13 with oligonucleotide βgal X4 successfully generated ES cells that produce enzymatically active β-galactosidase, as determined by X-gal staining (Figure 7a). Staining of untransfected ES cells or ES cells transfected with control

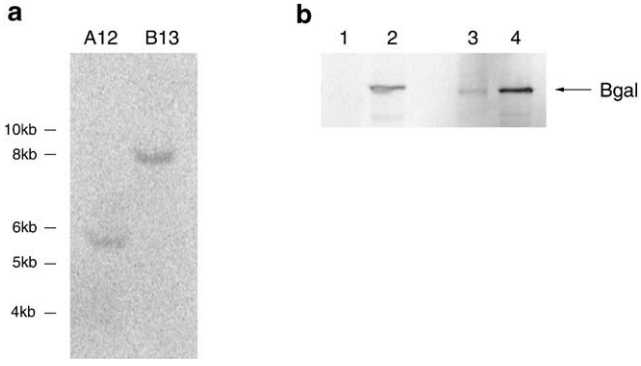

Figure 6 *Stable ES cell lines. ES cells lines with an integrated copy of the mutant* LacZ *gene were produced by transfection with linearized plasmid pCH110-G1651A-Neo. (a) Southern blotting demonstrates that cell lines A12 and B13 contain a single integrated copy of the mutant reporter gene. Since the restriction enzyme used to digest the DNA samples for blotting cuts the integrated vector only once, copies of the vector integrated at different locations in the genome produce different-sized bands on the Southern blot. (b) Western blotting shows that clones A12 (lane 3) and B13 (lane 4) produce mutant β-galactosidase protein. Controls include protein from untransfected ES cells (lane 1) and ES cells transiently transfected with the pCH110 plasmid (lane 2).*

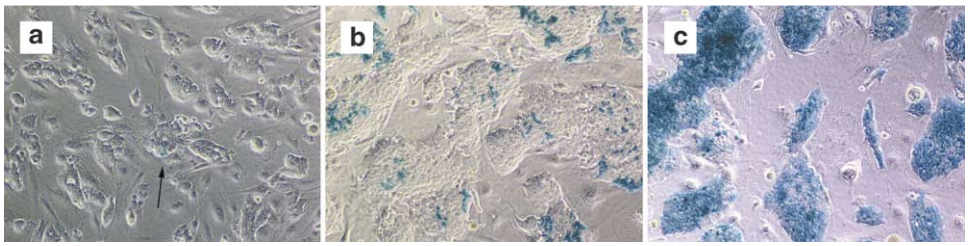

Figure 7 *Chromosomal correction in ES cells. ES cell clone A12, which has a single integrated copy of the mutant* LacZ *reporter gene, was transfected with oligonucleotide βgal X4. (a) X-gal staining 48 h following transfection demonstrated rare blue ES cells (arrow). Cells with a corrected* LacZ *gene were isolated by two rounds of cloning. (b) Blue ES cells were detected in a well from the first round of cloning, in which 50 transfected cells were plated per well in 96-well plates. (c) Following a second step of cloning by limiting dilution, several clones with a corrected* LacZ *gene were isolated. Essentially every cell expresses active β-galactosidase, and the ES cells remain undifferentiated.*

oligonucleotide βgal X5 produced no blue cells (data not shown). In 10 separate experiments, 2–12 (average 4) blue ES cells were detected following transfection of 2×10^5 ES cells in one well of a six-well plate, giving a conversion rate of approximately 1 in 5×10^4 cells. No blue cells were ever detected in control transfections. To verify that the observed correction of the *LacZ* mutation was permanent, ES cells were cloned by limiting dilution 48 h following transfection with correcting oligonucleotide. For the first round of cloning, 5×10^4 transfected mutant *LacZ* ES cells were plated in ten 96-well plates at a density of approximately 50 cells/well. As the cells approached confluence, they were split into two 96 well plates, and one set of plates was stained with X-gal. Blue ES cells were detected in two out of 960 wells (Figure 7b). The cells from these two wells were expanded from the second set of 96-well plates. The ES cells with active β-galactosidase were re-cloned by limiting dilution. Several corrected clones were obtained; an example is shown in Figure 7c. These results confirm that the oligonucleotide-directed correction of the mutant *LacZ* gene was permanent.

Discussion

Manipulation of DNA in mouse ES cells is a powerful approach for generating animal models of disease. The data presented here show that synthetic oligonucleotides can be used to create specific single-base alterations in the genomic DNA of mouse ES cells. Our results therefore indicate that oligonucleotides could be used to produce single-base changes in endogenous genes, thus allowing for the creation of accurate mouse models of inherited diseases, especially dominant ones. Mice with engineered single-base mutations could also be used to test disease-specific therapeutic approaches to gene therapy or gene correction. Finally, if the techniques described above can be transferred to use with human stem cells, it is possible that oligonucletoides could be used to correct mutations in stem cells from individual patients prior to using the cells for therapeutic purposes.

The efficiency of gene conversion observed using ssODN in CHO-K1 cells is similar to that reported.[30] In our experience, ssODN produced more base correction events than double-stranded chimeric oligonucleotides. This is not completely consistent with prior data, and may reflect the more consistent quality of the ssODN, which are easier to synthesize and purify than the original chimeric RNA–DNA oligonucleotides. As previously reported,[30,31] we observed that anti-sense oligonucleotides were more effective at base correction than sense oligos. We also observed that phosphorothioate-protected oligonucleotides may produce more gene conversion than 2′-O-methyl U protected oligonucleotides (Table 1). This may be due in part to the longer region of homology in phosphorothioate-protected DNA oligos, as βgal X3 with homologous 2′-O-methyl RNA for protection appeared to be slightly more active than βgal X1 with 2′-O-methyl U protection.

The actual frequency of base alteration observed in mouse ES cells was low, with both episomal and chromosomal reporter gene targets. Correction efficiency with the integrated *LacZ* reporter gene was on the order of 10^{-4}. This is approximately 10-fold lower than has been reporter for chromosomal correction in CHO cells.[30] A potential reason for this low correction efficiency is the low efficiency of ES cell transfection. We have found that electroporation and lipofection transfect approximately 5–10% of ES cells, when tested with an *EGFP* reporter gene (Ma *et al*, submitted). We have observed similar efficiencies of ssODN transfection into ES cells (data not shown). Consistent with this idea, data from the *in vitro* reaction showed that nuclear extracts from CHO-K1 and ES cells had similar gene conversion activity, while the efficiency of base conversion is 10-fold lower in transfected ES cells than CHO-K1 cells.[30] This difference is similar to the difference in transfection efficiency between CHO-K1 and ES cells.

While our current results demonstrate a low efficiency for base conversion in ES cells, there are several possible methods for increasing activity. First, different methods of delivering the ssODN to the ES cells could be evaluated. Several investigators have shown that direct microinjection of oligonucleotides or other molecules can increase activity of the injected compounds up to 1000-fold.[16] Second, longer pieces of DNA have also been used for specific base alteration, via what has been called short-fragment homologous replacement (SFHR). For example, 165 bp fragments of *Hprt* cDNA were used to correct a mutation in the *Hprt* gene in human lymphocytes.[37] More recently, 488 bp DNA fragments have been used to produce a 3 bp deletion in the *CFTR* gene in cultured airway cells.[38] Finally, adeno-associated virus (AAV) has also been used to deliver single-stranded DNA to cultured cells for gene conversion.[39]

Given that improvements in techniques will make use of oligonucleotides to produce mutations feasible in ES

cells, are there reasons to use oligonucleotides for gene targeting instead of standard techniques? There are several reasons that the answer is yes. Oligonucleotides are simple to synthesize and purify, whereas traditional gene targeting constructs require isolation and manipulation of large portions of genomic DNA from the locus of interest. Oligonucleotides could theoretically introduce a single-base mutation into an endogenous gene in a single step. In contrast, introduction of subtle mutations into genomic DNA using recombinase-mediated techniques, such as the Cre/lox system, requires at least two steps: one for targeting and one to excise the selective marker.[3] Doing the excision *in vitro* is faster, but excessive manipulation of ES cells in culture can decrease their pluripotency.[2] If mice expressing the desired recombinase in the appropriate tissue are available, the excision step can be done *in vivo*, but this requires extra breeding steps and thus additional time. In addition, it has been reported that Cre expression in cultured cells can produce chromosomal aberrations, possibly due to the presence of endogenous pseudo-*lox* sites in mammalian genomes.[10,40] This raises the concern that the phenotypes of mice produced using the Cre/lox system could be due, at least in part, to Cre-mediated mutations in addition to the planned knock-in allele.

There are limitations to the potential use of oligonucleotide-mediated gene targeting. Oligonucleotides can only introduce single-base changes into targeted genes, and are not capable of larger genetic alterations, or of conditional gene targeting approaches. Additional experiments will also be required to determine if oligonucleotides alter DNA sequences at sites other than the targeted gene, although no evidence of non-specific alterations has been reported to date.[21] Another major limitation is that oligonucleotides lack the advantage of incorporating selective markers into the targeting construct. The low rates of homologous recombination, on the order of 10^{-5}, observed with standard gene targeting vectors are overcome by the use of selective markers.[41,42] It may also be possible to use such selectable markers to enrich for ES cells that have undergone oligonucleotide-directed gene targeting. For example, it may be possible to use ES cells containing a mutant marker gene for oligo directed-targeting experiments. In such an experiment, ssODN designed to correct the defective marker would be co-transfected with ssODN designed to introduce the desired mutation into the gene of interest. Isolation of ES cell clones with an active selective marker may enrich for cells with the desired mutation. Experiments are in progress to test this approach.

If oligonucleotides were to be used for gene targeting in mouse ES cells, what level of base alteration would be needed to make the technique practical? The major limiting factor in isolating an ES cell clone with the desired mutation is detecting the single-nucleotide mutation in ES cell DNA. Since this would be similar to detecting a single-nucleotide polymorphism (SNP), there are now several efficient and high-throughput methods available for mutation detection. Examples include dot blotting, single-base extension assays, mass spectroscopic techniques and conformational methods such as SSCP and DHPLC. A mutation frequency of 0.1–1% would therefore make use of oligonucleotide-directed gene targeting feasible.

In summary, the use of oligonucleotides to introduce single-base mutations into endogenous genes in mouse ES cells provides an attractive approach to producing accurate animal models of inherited diseases. The primary advantage of such a technique is the ability to introduce a specific single-base change into a desired gene in a single step. In addition to introduction of specific mutations into known genes, it would also be possible to use oligonucleotides to introduce non-sense mutations into novel genes, in either ES cells or other cell types, as an alternative approach to disrupting gene expression. Further investigations are in progress to determine the full potential of this technique.

Materials and methods

Oligonucleotides

All oligonucleotides used in these experiments were synthesized by the Nucleic Acid Facility at the University of Pennsylvania. The resulting oligos were purified by denaturing electrophoresis on acrylamide gels as described.[19] Analytical gel electrophoresis of purified oligonucleotides demonstrated a single species of the correct size for each oligonucleotide used.

Plasmids

Plasmid pcDNA3-EGFP was produced by cloning the Xbal fragment containing the *EGFP* coding sequence from pEGFP (Clontech, Palo Alto, CA, USA) into the Xbal site of pcDNA3.1 (Invitrogen Life Technologies, Carlsbad, CA, USA). Clones with the EGFP insert in the correct orientation were identified by DNA sequencing. The desired mutations were introduced into pcDNA3-EGFP by PCR-based mutagenesis (Quik-Change; Stratagene, La Jolla, CA, USA). Following mutagenesis, clones with the desired mutations were identified by sequencing. Plasmid pCH110-G1651A-Neo was produced by cloning the neomycin resistance cassette from pcDNA3.1 into plasmid pCH110-G1651A.[28] Clones with the correct insert were identified by restriction analysis and verified by DNA sequencing.

Cell culture, transfection and selection

AB2.2 ES cells[43] were cultured on mitomycin C inactivated STO (ATCC, Manassas, VA, USA) or SNLP feeder cells, according to established protocols.[44] TL-1[45] and R1[46] ES cells were grown on inactivated mouse embryonic fibroblasts. CHO-K1 (ATCC) cells were cultured in F12 medium with 10% fetal calf serum.

CHO-K1 cells were plated at a density of 5×10^4 cells/well in six-well plates 18 h prior to transfection. CHO-K1 cells were transfected with 2 μg/well of reporter gene plasmids using 5 μg of Lipofectamine (Life Technologies, Carlsbad, CA, USA) plus 100 μg of CM9 peptide.[47] Prior to transfection, 0.8 ml of fresh complete medium was added to each well. The transfection mixtures, prepared in 0.2 ml of serum-free medium (Optimem, Life Technologies), were then added to the wells, and the plate centrifuged at $200 \times g$ for 5 min.[48] The transfection medium was removed after 4–6 h, and replaced with fresh medium for 1 h. Oligonucleotides (6 μg) were then transfected into the CHO-K1 cells overnight using the same method. The next day, the transfection medium was removed, and replaced with fresh medium. Cells

were assayed for reporter gene activity 48 h after starting the transfection.

ES cells were also transfected in six-well plates with Lipofectamine plus CM9 peptide using a total of 1 ml of medium per well. Except where noted, 2 μg of reporter plasmid and 6 μg of oligonucleotide were used per well; this corresponds to a molar ratio of plasmid:oligo of approximately 1:750. For transfections, ES cells were trypsinized and then 'panned' by plating them on gelatin-coated tissue culture dishes for 30–45 min to partially remove feeder cells. The panned ES cells were then pooled and counted. ES cells (2×10^5) in 0.8 ml of medium were then added to each well of a six-well plate that contained feeder cells. The transfection mixtures, prepared in 0.2 ml of serum-free medium (Optimem), were then added to the wells, and the plate centrifuged at $200 \times g$ for 5 min. Transfection medium was replaced with fresh media after 4–6 h. ES cells were assayed for reporter gene activity 48 h after transfection.

To produce ES cells with an integrated copy of the mutant *LacZ* reporter gene, 2×10^6 AB2.2 ES cells were transfected with 15 μg of linearized pCH110-g1651a-Neo plasmid. Stable transfectants were selected with 300 μg/ml of G418 in ES cell media for 10 days. The G418-resistant ES cell colonies were isolated and expanded in 24-well plates according to standard techniques.[44] ES cell clones were screened for the presence of the mutant *LacZ* gene by Southern blotting.[44,49] Clones with single integrated copies of the mutant reporter gene were further screened by Western blotting using a monoclonal anti-β-galactosidase antibody (Promega) to identify cell lines that produced the mutant β-galactosidase protein.

For gene conversion experiments using the mutant *LacZ* ES cells, 15 μg of ssODN were transfected into the stable ES cell lines using 45 μl of Oligofectamine (Life Technologies) and 350 μg CM9 peptide. To clone corrected *LacZ* ES cells, 5×10^4 transfected mutant *LacZ* ES cells were plated in ten 96-well plates at a density of approximately 50 cells/well. As these cells approached confluence, each plate was split into two 96-well plates. One set of these plates was then stained with X-gal. Cells from these wells with blue cells were expanded from the second set of 96-well plates. The ES cells with active β-galactosidase were re-cloned by limiting dilution in 96-well plates. Cells from this second cloning step were also split into duplicate 96-well plates prior to staining with X-gal.

In vitro assay

Nuclear extracts were prepared from cells grown in log phase as described.[28] Extracts were assayed for gene-conversion activity using the mutant *LacZ* reporter plasmid, also as described.[28] Briefly, reporter plasmid and oligonucleotide were incubated in nuclear extract for 1 h. The plasmid DNA was extracted, and one-tenth of the DNA from each reaction was used to transform electro-competent P90C bacteria. The bacteria were plated on LB agar plates containing X-gal (100 μg/ml) and ampicillin (50 μg/ml). The number of blue colonies and total colonies were recorded.

β-galactosidase activity

β-galactosidase activity in cultured cells was detected as described.[30] The use of pH 8 inhibited endogenous mammalian β-galactosidases. Blue cells with normal morphology were counted as positive. Percent gene conversion in experiments with CHO cells was determined by dividing the number of blue cells by the number of cells plated in the wells. Cells were examined and photographed in culture dishes by light and fluorescence microscopy using a Nikon Diaphot or Nikon TE300 inverted microscope equipped with a color digital camera.

FACS

For fluorescence activated cell sorting (FACS) analyses, cells were trypsinized, resuspended and PBS and kept on ice. FACS was performed in the Cell Sorting Core Facility at the University of Pennsylvania using a Becton Dickenson FACScan instrument. CellQuest software was used to acquire and analyze FACS results. The percent of gene conversion was determined by the number of live (gated) cells with fluorescence above background.

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