Stimulation of Oligonucleotide-Directed Gene Correction by Redβ Expression and MSH2 Depletion in Human HT1080 Cells

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The correction of disease-causing mutations by single-strand oligonucleotide-templated DNA repair (ssOR) is an attractive approach to gene therapy, but major improvements in ssOR efficiency and consistency are needed. The mechanism of ssOR is poorly understood but may involve annealing of oligonucleotides to transiently exposed single-stranded regions in the target duplex. In bacteria and yeast it has been shown that ssOR is promoted by expression of Redβ, a single-strand DNA annealing protein from bacteriophage lambda. Here we show that Redβ expression is well tolerated in a human cell line where it consistently promotes ssOR. By use of short interfering RNA, we also show that ssOR is stimulated by the transient depletion of the endogenous DNA mismatch repair protein MSH2. Furthermore, we find that the effects of Redβ expression and MSH2 depletion on ssOR can be combined with a degree of cooperativity. These results suggest that oligonucleotide annealing and mismatch recognition are distinct but interdependent events in ssOR that can be usefully modulated in gene correction strategies.

INTRODUCTION

Many inherited genetic disorders are caused by simple mutations in single disease genes. Correction of these mutations is an attractive approach to gene therapy with several advantages over gene addition approaches (Humbert et al., 2012; Yáñez and Porter, 1998). Gene correction can be achieved by homologous recombination (HR) with a double stranded DNA (dsDNA) repair template including several kilobase pairs of homology to the target locus. This general approach is widely used for site-specific mutagenesis in mammalian cells (gene targeting), frequencies of which are typically in the region of $10^{-3}$ to $10^{-4}$ events per transfected cell (Deng and Capecchi, 1992). Fortunately frequencies can be greatly stimulated by use of customised endonucleases to make a double strand break (DSB) in the target locus (Gaj et al., 2013; Umov et al., 2005), or vectors based on Adeno-Associated Virus (Khan et al., 2011). Limitations remain, however, including off-target cleavage by the nucleases, competing DSB repair by mutagenic nonhomologous end-joining, and the challenges of constructing and efficiently delivering both custom nucleases and large DNA templates. Single-stranded oligonucleotides can also be used as templates for chromosomal gene modification and have the attraction of being relatively easy to make and deliver. Initial work with chimeric RNA/DNA oligonucleotides (RDOs) (Andersen et al., 2002; Igoucheva et al., 2004; Liu et al., 2003) was difficult to reproduce (Manzano et al., 2003; Taubes, 2002) and has been superseded by gene modifications mediated by single-stranded oligodeoxyribonucleotides (ssOs) (Aarts and te Riele, 2011). Nevertheless, unaided frequencies of ssO-mediated gene repair/modification (ssOR) are still variable and often very low (Aarts et al., 2006; Hu et al., 2005; Igoucheva et al., 2001; Kenner et al., 2002; Nickerson and Colledge, 2003; Pierce et al., 2003).

Although ssOR, like gene targeting with dsDNA templates, can be greatly enhanced by nucleotide-mediated cleavage of the target locus (Chen et al., 2011; Majumdar et al., 2008; Radecke et al., 2006; 2010), and seems to involve some components of the HR pathway of DSB repair (Ferrara and Kmiec, 2004; McLachlan et al., 2009; Morozov and Wawrousek, 2008), an attractive model for ssOR involves annealing of the ssO to regions of target ssDNA transiently exposed during DNA replication (Aarts and te Riele, 2011; Papaioannou et al., 2012). Consistent with this, procedures likely to promote or stabilise ssO annealing at replication forks can enhance ssOR without the need to risk introducing a chromosomal break. For example, ssOR can be stimulated by increasing the proportion of cells with replication forks and so increase opportunities for ssO annealing (Brachman and Kmiec, 2005; Olsen et al., 2005; Wu et al., 2005). Furthermore, cells deficient in the DNA mismatch repair (MMR), a key protein of the DNA mismatch repair (MMR) pathway (Jiricny, 2006), support greatly elevated levels of ssOR (Dekker et al., 2003), most likely because annealed ssOs, which must have some mismatches in order to modify the chromosome, are no longer removed by MMR. To exploit the

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latter observation it is important to show that transient depletion of MSH2 also stimulates ssOR.

Another approach that may promote ssO annealing, and therefore ssOR, is expression of Redβ, a single strand DNA annealing protein encoded by bacteriophage lambda (Efer et al., 2000; Iyer et al., 2002). Expression in E. coli of Redβ, and its partner Redα, a 5′-3′ exonuclease (or the equivalent rac prophase proteins, RecE and RecT), is the basis of a range of genetic engineering methods termed “recombinering” that require only short regions of homology between recombining dsDNA partners (Court et al., 2002; Muyres et al., 2000). Recombinering also encompasses the use of ssOls to introduce defined sequence alterations into a target duplex, a procedure known to be particularly efficient and to depend on Redβ but not on Redα (Ellis et al., 2001; Swaminathan et al., 2001; Zhang et al., 2003). In common with ssOR in mammalian cells, Redβ-mediated ssOR in E. coli is impaired by the MMR system (Costantino and Court, 2003; Li et al., 2003). Furthermore, both mammalian ssOR (Igoucheva et al., 2001) and Redβ-mediated ssOR in E. coli (Ellis et al., 2001; Swaminathan et al., 2001; Zhang et al., 2003) can display a strand preference. These parallels between ssOR in mammalian cells and Redβ-mediated ssOR in bacteria suggest that Redβ might be able to promote ssOR in mammalian cells, a possibility that was supported by preliminary analyses in mouse ES cells (Zhang et al., 2003).

To characterise the effects of Redβ in mammalian cells in detail we describe here a human cell line in which nuclear expression of Redβ can be induced by removal of tetracycline from the medium. We show that such induction does indeed stimulate ssOR, that this effect can be enhanced by concomitant MSH2 depletion and that these stimulations are co-operative.

MATERIALS AND METHODS

Cell culture

The human fibrosarcoma HT1080 cell line was from American Tissue Culture Collection, grown as previously described (Itzhaki et al., 1997) in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal calf serum (GIBCO BRL, USA). Where appropriate the following antibiotics were added to the medium: zeocin (200 μg/ml), hygromycin (200 μg/ml), G418 (300 μg/ml) and tetracycline (1 μg/ml) (Sigma-Aldrich Inc., USA). Rh14 cells are zeocin-resistant HT1080 derivatives, stably transfected with a plasmid encoding a tetracycline transactivator protein. Unless stated otherwise, Rh14 cells transfected with pTRE-Tight-Redβ were maintained with tetracycline in the medium.

Plasmids and oligonucleotides

pcDNA/PGK-neo* and pcDNA-Redβ/PGK-neo* have been described (Zhang et al., 2003). A derivative of the latter, pcDNA-Redβ/PGK-neo*, was made by adding AGGATCCCAATTGGCCCTAAAAGAGCGTAAAATCGCAGTCACCTGAT in place of AGGATCCCAATTGGAGT anticodon of Redβ. To make pTRE-Tight-Redβ, the nls-Redβ open-reading-frame was removed from pcDNA-Redβ/PGK-neo* as an 825 bp BamHI/HindIII fragment and cloned into the multiple cloning site of pTRE-Tight (BD Biosciences, USA). Oligonucleotides were commercially synthesised by Sigma-Aldrich Inc.

Stable transfection

Stable transfection was performed by electroporation at 400 V and 250 μF on a BIO-RAD (USA) Gene Pulser as described (Itzhaki et al., 1997). HT1080 cells containing humanised transactivator (hTATA) (8 × 10^6 cells) were co-transfected with pTRE-Tight-Redβ and pcDNA-Hyg-PGK-neo* (10 μg total, 20-fold molar excess of pTRE-Tight-Redβ) by electroporation; selection in hygromycin started 48 h after transfection and continued for 10-14 days until hygromycin-resistant colonies appeared.

Immunoblots

Immunoblots were as described (Liu et al., 2012). Briefly, cell pellets were prepared in lysis buffer (200 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, The β-glycerophosphate, EDTA, Na3VO4, leupeptin). Lysates were electrophoresed on SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h at room temperature with 5% milk protein, 0.1% Tween 20 in PBS, then probed with antibodies. Redβ was detected with a rabbit polyclonal antibody used at a 1:1000 dilution. MSH2 was detected with mouse monoclonal antibody (Calbiochem, USA), used at a 1:100 dilution. Actin was detected with rabbit polyclonal antibody (SIGMA-Aldrich), used at a 1:1000 dilution. Secondary antibodies were horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (DAKO, USA), used at a 1:2000 dilution, or HRP-conjugated goat anti-mouse antibody (DAKO), used at a 1:1000 dilution.

Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with 2% (v/v) paraformaldehyde in PBS for 15 min, and then permeabilised with 0.4% (v/v) Triton X-100 in PBS for 15 min. Coverslips were washed with PBS and blocking buffer (5% (v/v) fetal bovine serum, 5% (v/v) normal goat serum in PBS) and then incubated for 1 h each with primary (Redβ) polyclonal and secondary (FITC-conjugated donkey anti-rabbit IgG; Autogen Bioclear, U K) antibodies, diluted (1:100) in blocking buffer. Coverslips were washed with PBS and mounted with Vectashield mounting medium containing DAPI (VECTOR laboratories, USA). Slides were viewed with a fluorescence microscope (Leica DM RB, USA).

Single-strand oligonucleotide repair

The neo*-carrying plasmid and ssO were co-transfected by lipofectamine 2000 (Invitrogen, USA) according to manufacturer’s instructions. Three days before ssO transfection, tetracycline was removed from (- Tet, Redβ+) or, maintained in (+ Tet, Redβ-) the medium; absence or presence of tetracycline in the medium was maintained for the following four days before re-storing tetracycline to all media. The day before ssO transfection, either untreated cells, or cells that had been transfected with siRNA for 48 h, (next section), were seeded at ~50% confluence in 15 cm diameter dishes. The following day, a mixture (3 ml) containing pcDNA-Hyg-PGK-neo* (5 pmole, 24 μg), ssO (95 pmole, 2 μg) and lipofectamine 2000 (60 μl) in OptiMEM was prepared according to manufacturer’s instructions, and added to fresh growth medium (9 ml) in each 15 cm diameter dish of cells, now approaching confluence (3 × 10^6 per dish). After 24 h, the cells of each dish were detached by trypsinisation and distributed into three similar dishes in medium containing G418 and tetracycline. On occasion, a sample of...
cells was taken at this stage for immunoblot analysis of Redβ expression. Fresh medium containing G418 and tetracycline was added every 2-3 days for 10-14 days until colonies appeared. Colonies were stained and counted as described (Porter and Itzhaki, 1993).

**siRNA transfection**

Sequences of siRNA duplexes (Dharmacon Inc, USA) used are shown in Supplementary Table S1. Four of these were components of a "SMARTpool" mixture of duplexes based on the MSH2 coding sequence. The siRNAs were delivered by oligofectamine (Invitrogen) according to manufacturer's instructions. Cells grown in medium with tetracycline were plated at ~50% confluence, (2 million cells per 15 cm diameter dishes) maintaining tetracycline. The following day, a mixture (1.8 ml) containing siRNA (90 μl of 20 μM stock) and oligofectamine (90 μl) in OptiMEM was prepared according to manufacturer's instructions, and added to each 15 cm diameter dish of cells, now approaching confluence, containing 8 ml medium (with or without tetracycline), to give a final siRNA concentration of 180 nM. After 48 h, the cells of each dish were trypsinised and distributed into two 15cm diameter dishes (for ssOR) and/or at a similar density into a single well of a 6-well plate (for immunoblot analysis). After a further 24 h, cells were processed for ssOR (see previous section) or harvested for immunoblots.

**RESULTS**

**Inducible Redβ expression in HT1080 cells**

Because Redβ expression could be deleterious in mammalian cells, we expressed Redβ stably but inducibly. The Redβ open-reading-frame was cloned, with an N-terminal nuclear localisation signal (nls), downstream of a tetracycline responsive element (TRE) to generate pTRE-Redβ (Fig. 1A). To provide a target gene for ssOR, as well as a drug resistance marker for the selection of stable transfectants, a second plasmid, pcDNA/PGKneo* was used (Fig. 1B). It carries two expression cassettes, one conferring resistance to hygromycin, the other (PGKneo*) capable of conferring resistance to G418 only after correction of a 4 bp insertion in its neomycin phosphotransferase (neo) coding sequence. The DNA sequence of this region, and of repair and control ssOs used in ssOR assays, are shown in Figs. 1C and 1D. Plasmids pTRE-Redβ and pcDNA/PGKneo* were co-transfected, the former in molar excess, into an HT1080 derivative [Rht14; (Brough et al., 2011)] expressing an improved tetracycline transactivator protein capable of driving transcription from the TRE. Hygromycin-resistant colonies were expanded and analysed for Redβ expression by immunoblotting. Several clones showing tetracycline-regulated Redβ expression were identified and immunoblots for some are shown in Fig. 1E.

A detailed analysis of one of these clones (Rβ[1.6]) showed that, after addition of tetracycline to the growth medium, almost all immunoblot-detectable Redβ expression was lost within a period of 4 days (Fig. 2A). Removal of tetracycline from such cells resulted in abundant Redβ re-expression within 2 days and close to maximal expression within 3 days. Expression of Redβ had no effect on the doubling time of clone Rβ[1.6] over a period of 17 days (Fig. 2B), at the end of which Redβ expression was still abundant in the culture that had been grown without tetracycline throughout (Fig. 2C). There were thus no acutely adverse effects of Redβ expression on cellular proliferation. Immunofluorescence on clone Rβ[1.6] confirmed that Redβ was located in the nucleus (Fig. 2D).

**Stimulation of gene correction by Redβ**

To measure ssOR of a chromosomal neo* target, cells from each of three clones with inducible Redβ (Rβ[1.2], Rβ[1.6] and Rβ[1.8]) were transfected with a 70nt repair ssO (RO, Fig. 1D).

![Fig. 1. Reagents used for Redβ expression and ssOR assays. (A) Structure of Redβ expression plasmid. (B) Structure of plasmid carrying the neo* target for gene correction. (C) Sequence surrounding the 4 bp insertion (bold underlined) in neo*. (D) Sequence of control (CO) and repair (RO) ssOs used in ssOR assays. (E) Immunoblot analysis of Redβ expression in hygro5 clones isolated after co-transfection of HT1080 cells with the plasmids shown in (A) and (B). Clones were expanded and grown continually with tetracycline (Tet) in the growth medium (+), or for 3 days after the removal of tetracycline (-), before analysis.](http://molcells.org)
Correction of the neo\textsuperscript{*} gene should generate G418\textsuperscript{r} colonies, but none was obtained from a total of 16 million transfected R\textit{j}1.6 cells, and 3 million transfected R\textit{j}1.2 or R\textit{j}1.8 cells. The frequency of gene correction for the chromosomal neo\textsuperscript{*} was therefore well below 1 event per 10\textsuperscript{6} cells. For all further measurement of ssOR we therefore measured gene correction of an episomal neo\textsuperscript{*} gene by co-transfection of pcDNA/PGKneo\textsuperscript{*} with measurement of ssOR frequencies were calculated from frequencies of G418 colonies as described in “Materials and Methods”; parallel control experiments using CO in place of RO generated no G418-resistant colonies; means and standard deviations of ssO frequencies are shown for three independent experiments; numerical fold increases and significance (\textasternot indicates p-values of < 0.05; Student’ \textit{t}-test) are shown.

To determine whether transient Red\textit{j} expression could be used to stimulate ssOR, parental HT1080 cells were co-transfected with a neo\textsuperscript{*} expression vector and ssO with or without Red\textit{j} expression vector (Supplementary Table S2). Although immunoblotting confirmed Red\textit{j} expression in amounts similar to stably transfected cells, any Red\textit{j}-mediated increase in ssOR was too small to detect in this experiment.

**Stimulation of gene correction by MSH2 knockdown with and without Red\textit{j} expression**
Control or MSH2-specific siRNAs were delivered to parental HT1080 cells with oligofectamine 72 h before co-transfecting the target neo\textsuperscript{*} gene with repair or control ssOs. Cell samples taken for immunoblot analysis at the time of ssOR induction showed clear MSH2 depletion specifically by the MSH2 siRNA (Fig. 2E). Subsequent selection in G418 and scoring for G418\textsuperscript{r} colonies revealed a nearly 2-fold increase in the frequency of ssOR in MSH2-depleted cells (Fig. 3B).

To gain further evidence that transient MSH2 depletion can stimulate ssOR, and to test whether this could be enhanced by the simultaneous induction of Red\textit{j} expression, three independent MSH2 depletion experiments were carried out in clone

R\textit{j}1.6 cells, with or without the induction of Red\textit{j}, measuring neo\textsuperscript{*} correction throughout. Again, immunoblot analysis of samples taken at the time of the neo\textsuperscript{*} and ssO co-transfection confirmed MSH2 depletion (results for one experiment are shown in Fig. 2E). The frequencies of G418\textsuperscript{r} colonies obtained are summarised in Fig. 3C. In these experiments the individual effects of Red\textit{j} expression and MSH2 depletion were observed again, though were slightly less pronounced than in the previ-
ous experiments (Figs. 2A and 2B). Notably, however, the stimulation of ssOR by Redβ expression became more pronounced when MSH2 was depleted than when it was not (2.4-fold vs. 1.5-fold). Similarly, the stimulation of ssOR by MSH2 depletion became more pronounced when Redβ was induced than when it was not (2.3-fold vs. 1.5-fold). Furthermore, the overall stimulation of ssOR caused by the combination of Redβ expression and MSH2 depletion was 3.6-fold, a figure considerably larger than the product of the individual stimulations (2.3 \( \times \) 1.5 \( \times \) 1.5). These observations suggest that the two stimulatory mechanisms may in fact work co-operatively.

**DISCUSSION**

The use of Redβ expression for genetic manipulations in bacteria is well established, but the suggestion (Swamnathan et al., 2001; Zhang et al., 2003) that this may be extended to eukaryotic organisms has received little attention. We have therefore begun to study the effects of nuclear Redβ expression in a human cell line concentrating, in the present study, on ssOR. We find that Redβ carrying an N-terminal NLS is well tolerated in human fibroblast cells and able to stimulate ssOR on co-transfected episomes. Although the degree of stimulation is small (< 2-fold), it is highly reproducible and, in common with Redβ-mediated ssOR in bacteria (Costantino and Court, 2003; Li et al., 2003), it can be enhanced by inactivation of the MMR system. The ssOR activity of Redβ in mammalian cells therefore appears to be qualitatively similar to that in bacterial cells raising the possibility that quantitatively useful stimulations may yet be achievable in mammalian cells.

The mechanism of ssOR is believed to involve annealing of the ssOs to regions of ssDNA in the target locus transiently exposed during replication (Court et al., 2002; Dekker et al., 2003; Li et al., 2003; Zhang et al., 2003) or transcription (Igoucheva et al., 2003). We suggest that Redβ is able to promote such ssO annealing, albeit modestly, in mammalian cells. MMR has previously been proposed to suppress ssOR by recognising and removing imperfectly annealed ssOs (Costantino and Court, 2003; Dekker et al., 2003). The fact that we observed some co-operativity between Redβ expression and MSH2 depletion, suggests that annealing and mismatch recognition may be concerted events during ssOR. It is possible, for instance, that Redβ protects annealed ssOs from the MMR system, while impaired MMR slows replication forks allowing Redβ to promote further annealing.

There are several reasons why the extent of Redβ-dependent ssOR activity may be limited in mammalian cells. First, one or more cellular proteins may compete or otherwise interfere with the ability of Redβ to access ssDNA. Proteins that, like Redβ, have single strand DNA binding or annealing activity may be particularly relevant here, e.g. Replication Protein A (Bochkarev and Bochkareva, 2004), Rad52 (Listby and Rothstein, 2009). Transient depletion of such candidate proteins by RNA interference could be used to explore this possibility. Second, ssO instability may be a limiting factor. Although DNA stability is not a major factor for recombining with ssOs in bacteria, it is limiting for recombining with duplex DNA where expression of Redβ is required to inactivate host nuclease activities. To stabilise ssOs during ssOR experiments in mammalian cells, 2′-O-methyl-uracil residues are often added to 5′ or 3′ ends of ssOs, but their efficacy in this context is questionable although other modifications may help (Andrieu-Soler et al., 2005; Dekker et al., 2003). Other aspects of ssO design, such as length and mismatch position, are also known to be important variables. It may therefore be that larger stimulations can be achieved after ssO design has been re-optimised in the context of Redβ-mediated ssOR. Alternatively, cellular nucleases with known specificity for ssDNA (Shevelev and Hübscher, 2002) might usefully be depleted by RNA interference. One approach that may circumvent both ssO instability and preferential binding of endogenous proteins may be to co-express Redβ with Redβ and to use dsOs in place of ssOs. This is suggested by the fact that Redβ is thought to physically interact with Redβ so that the generation of 3′- ssDNA tails by exonuclease digestion occurs cooperatively with the formation of Redβ-ssDNA filaments (Court et al., 2002; Muyrers et al., 2000). Lastly, it is conceivable that bacterial host proteins interact with Redβ to generate maximum ssOR activity, and that similar proteins are either absent in mammalian cells or too poorly conserved to interact effectively. Although we cannot discount this possibility, it is notable that strand annealing (and invasion) reactions are catalysed by Redβ in vitro without the need for accessory proteins (Karakousis et al., 1998; Li et al., 1998).

Our results go beyond a previous study (Zhang et al., 2003) in which mouse ES cells were stably transfected with pcDNA-redβ/PGK-neo; in contrast to cells transfected with vector only, these gave rise to G418R colonies at low frequency when transfected with a repair ssO. However subsequent analysis showed that Redβ was predominantly cytoplasmic in these cells and attempts at similar experiments involving nls-Redβ (i.e. using pcDNA-nlsredβ/PGK-neo) failed to generate clones with nuclear Redβ, presumably because nuclear Redβ is not tolerated by ES cells. It is unclear at present why HT1080 cells should tolerate Redβ expression in the nucleus better than ES cells. ES cells have a short doubling time and it is possible that this makes them more sensitive to perturbations in the DNA repair machinery. Alternatively, a checkpoint mechanism may be inactive in HT1080 cells that, in ES cells, is responsible for sensitivity to Redβ expression. Redβ does appear to be tolerated in yeast where delivery of a Redβ expression construct stimulated ssOR nine-fold (Brachman and Kmiec, 2003).

Our results with MSH2 depletion extend a previous study (Dekker et al., 2003) showing that ES cells in which both MSH2 alleles are disrupted support up to 100-fold greater levels of ssOR than control cells. Here we have shown that transient MSH2 depletion by RNA interference can be used to promote ssOR, a necessary advance if efforts to stimulate ssOR are to be used more widely and, especially, for therapeutic purposes. The stimulations achieved were relatively modest but might be improved by the use of more efficient siRNA, or by the combined use of siRNA specific for more than one DNA mismatch repair protein.

**Note:** Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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