ARTICLES

Highly efficient endogenous human gene correction using designed zinc-finger nucleases

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Permanent modification of the human genome *in vivo* is impractical owing to the low frequency of homologous recombination in human cells, a fact that hampers biomedical research and progress towards safe and effective gene therapy. Here we report a general solution using two fundamental biological processes: DNA recognition by C_2H_2 zinc-finger proteins and homology-directed repair of DNA double-strand breaks. Zinc-finger proteins engineered to recognize a unique chromosomal site can be fused to a nuclease domain, and a double-strand break induced by the resulting zinc-finger nuclease can create specific sequence alterations by stimulating homologous recombination between the chromosome and an extrachromosomal DNA donor. We show that zinc-finger nucleases designed against an X-linked severe combined immune deficiency (SCID) mutation in the *IL2R*γ gene yielded more than 18% gene-modified human cells without selection. Remarkably, about 7% of the cells acquired the desired genetic modification on both X chromosomes, with cell genotype accurately reflected at the messenger RNA and protein levels. We observe comparably high frequencies in human T cells, raising the possibility of strategies based on zinc-finger nucleases for the treatment of disease.

Most human monogenic disorders remain difficult to treat because therapeutic transgenes do not undergo homologous recombination (HR) into the mutated locus[1,2], and gene addition by virus-driven random integration remains a challenge owing to transgene silencing, improper activity or misintegration[3,4]. Furthermore, targeted alteration of DNA sequence *in vivo*—in principle, a powerful basic research technique for studying genome function—in mammals requires sophisticated targeting vectors and drug-based selection[1,2], which limits the use of this approach[5–7].

The C_2H_2 zinc-finger, originally discovered in *Xenopus*[8], is the most common DNA binding motif in all metazoa[9]. Each finger recognizes 3–4 base pairs of DNA via a single α-helix[10,11], and several fingers can be linked in tandem to recognize a broad spectrum of DNA sequences with high specificity[12–14]. Engineered zinc-finger protein (ZFP)-based DNA binding domains with novel specificities have been extensively applied *in vivo* to target various effector domains[12,15]. Work from the Chandrasegaran laboratory has shown that a ZFP can be coupled to the nonspecific DNA cleavage domain of the Type IIS restriction enzyme, *Fok*I, to produce a zinc-finger nuclease (ZFN)[16], which then cuts the DNA sequence determined by the ZFP[16,17]. An important specificity mechanism derives from the requirement that two ZFNs bind the same locus, in a precise orientation and spacing relative to each other, to create a double-strand break (DSB; Fig. 1a)[17]. One mechanism by which eukaryotic cells heal DSBs is homology-directed repair (Fig. 1b)[18–20], which transfers information missing at the break from a homologous DNA molecule (Fig. 1b). Work from the Jasin laboratory[21], followed by that of others[22,23], demonstrated that the endonuclease I-*Sce*I can potentiate HR into loci previously engineered to contain its own recognition site, and the Carroll[24,25] and Baltimore[26] laboratories have shown that a ZFN-invoked DSB increases the rate of HR in model systems.

Here we invoke this process at an endogenous locus in the human genome. We show that a DSB induced by engineered ZFNs at an inherited disease mutation hotspot rapidly leads to permanent, precise modification of up to 20% of the chromatids. Such unprecedented HR frequency, combined with the ability to engineer ZFNs against essentially any sequence, establish the usefulness of ZFN-driven genome editing as a tool for human somatic cell genetics, and also illuminate the potential for gene correction therapy of human inherited disease.

Optimization of ZFN-driven gene correction

To rapidly gauge the potential for ZFN-driven alteration of the genome *in vivo*, we elaborated on published work[26] and established a reporter system for gene correction (Fig. 1c). We used an archive of engineered zinc-finger motifs[15] to assemble two ZFNs against the gene encoding enhanced green fluorescent protein (eGFP). The DNA stretch between the ZFP binding sites was then disabled by a stop codon and a frameshift, and the resulting nonfunctional transgene was stably integrated at a single location (data not shown) in the genome of HEK 293 cells. When a fragment of wild-type eGFP corresponding to the mutation-disabled stretch was introduced into this reporter cell line as plasmid DNA, approximately 1–2 cells in 500,000 restored GFP function (left column of Fig. 1c: donor only), as expected on the basis of published measurements[2]. In contrast, four days after the simultaneous introduction of the donor and ZFN expression constructs, 2.2% of asynchronously growing cells and 10.2% of cells arrested for 30 hours at the G2/M cell cycle boundary were GFP-positive (right column of Fig. 1c: donor + ZFNs). These

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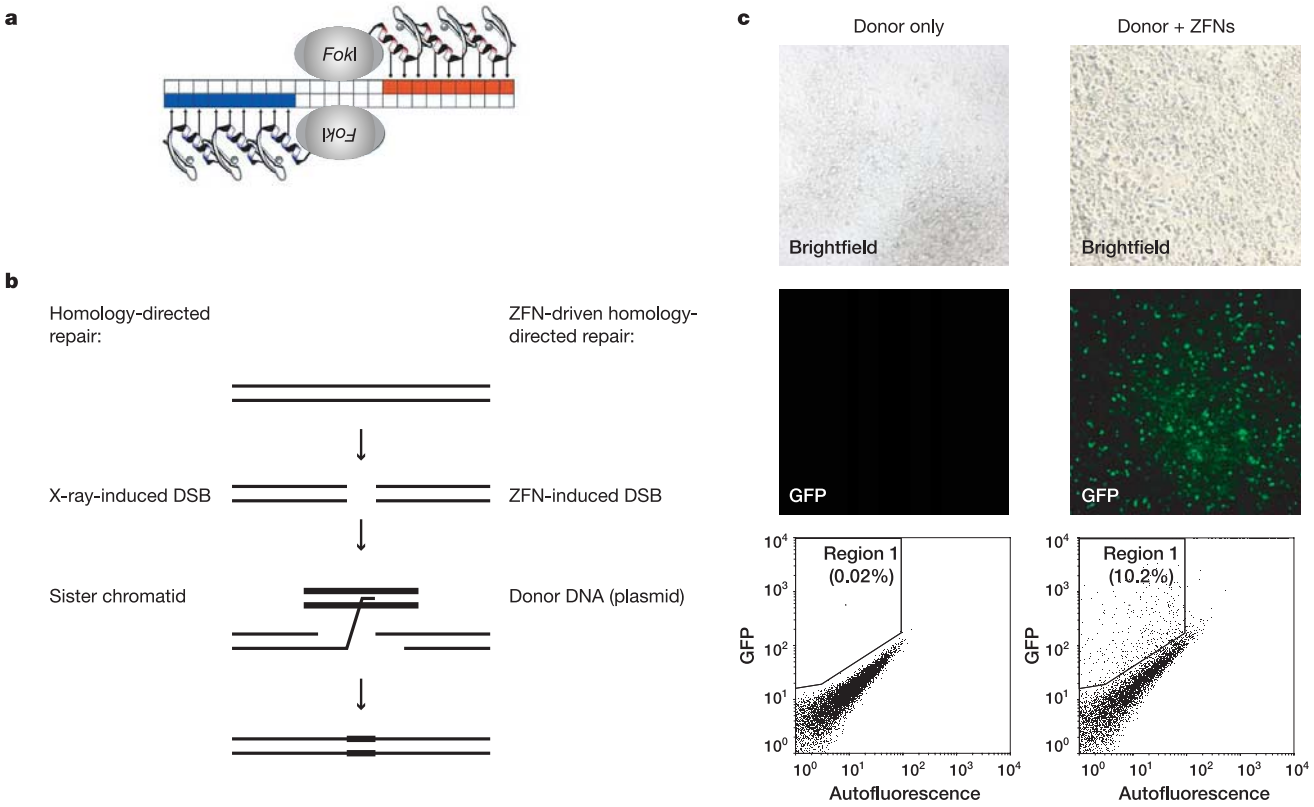

Figure 1 | Designed ZFNs enable correction of a chromosomal reporter gene in over 10% of the cells. a, Schematic of a DNA-bound ZFN pair. **b**, Homology-directed repair via 'short-patch gene conversion' of a DSB induced by X-rays using genetic information from a sister chromatid (left side) or of a site-specific DSB created by ZFNs, using a plasmid donor (right side). **c**, Cells carrying a mutated GFP reporter were transiently transfected with a donor plasmid carrying a fragment of wild-type GFP (left column), or the donor plasmid and the ZFNs (right column). Cells were arrested at G2/M before release and analysed by fluorescence-activated cell sorting (FACS) for GFP with GFP-positive cells bracketed in region 1.

data indicated that ZFNs could be used in a selection-free scheme to increase the rate of HR at a chromosomal reporter gene by five orders of magnitude.

Targeted alteration of DNA sequence at a human disease locus

Our ultimate goal is gene correction therapy for human disease, so we set out to determine whether ZFNs can evoke a comparable increase in HR frequency at an endogenous gene and focused our efforts on the *IL2Rγ* gene (the protein product is known as γC), mutations in which cause X-linked SCID[27]. Maximal HR frequency is observed when a DSB is evoked close to the mutated site[28], and so we used our ZFP archive[29] to assemble two DNA-binding domains, each containing four zinc-finger motifs and thus recognizing a total of 24 base pairs (bp) surrounding an X-linked SCID mutation hotspot

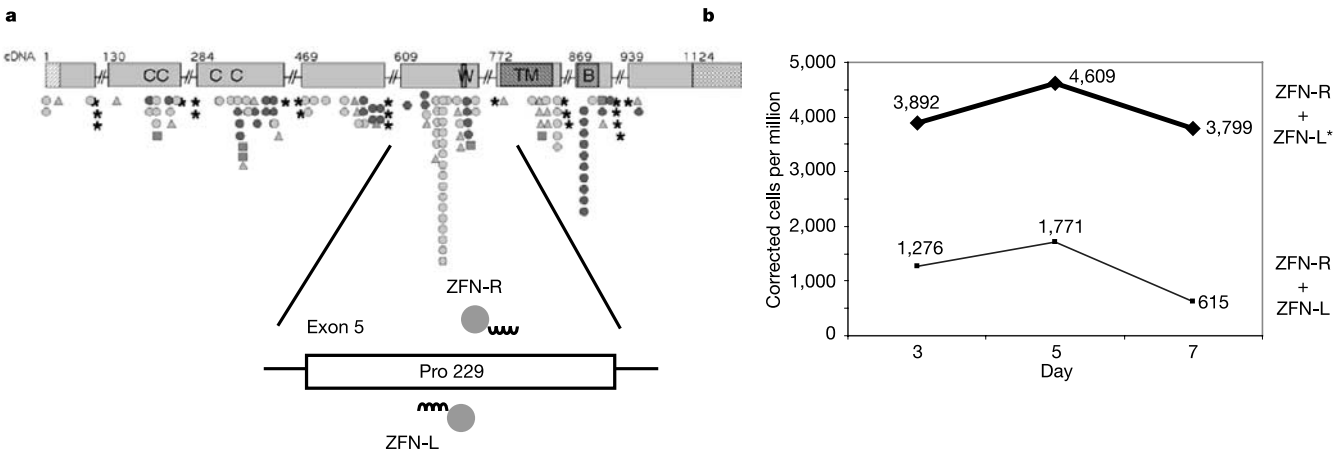

Figure 2 | Design and optimization of ZFNs directed against the X-linked SCID mutation hotspot of *IL2Rγ*. a, Map of the human *IL2Rγ* gene, with positions of disease-causing mutations (http://genome.nhgri.nih.gov/scid/IL2RGbase.shtml)[30] and the the ZFN binding sites annotated. **b**, ZFP optimization (upper line) markedly improves *in vivo* gene correction frequency of a chromosomal GFP reporter gene disabled by insertion of a fragment of the *IL2Rγ* gene carrying the ZFN recognition sites. FACS quantitation of the number of GFP-positive cells 3, 5, and 7 days post-transfection comparing archive-derived (lower line) or optimized (upper line, ZFN indicated by asterisk) ZFNs transfected along with a GFP donor plasmid.

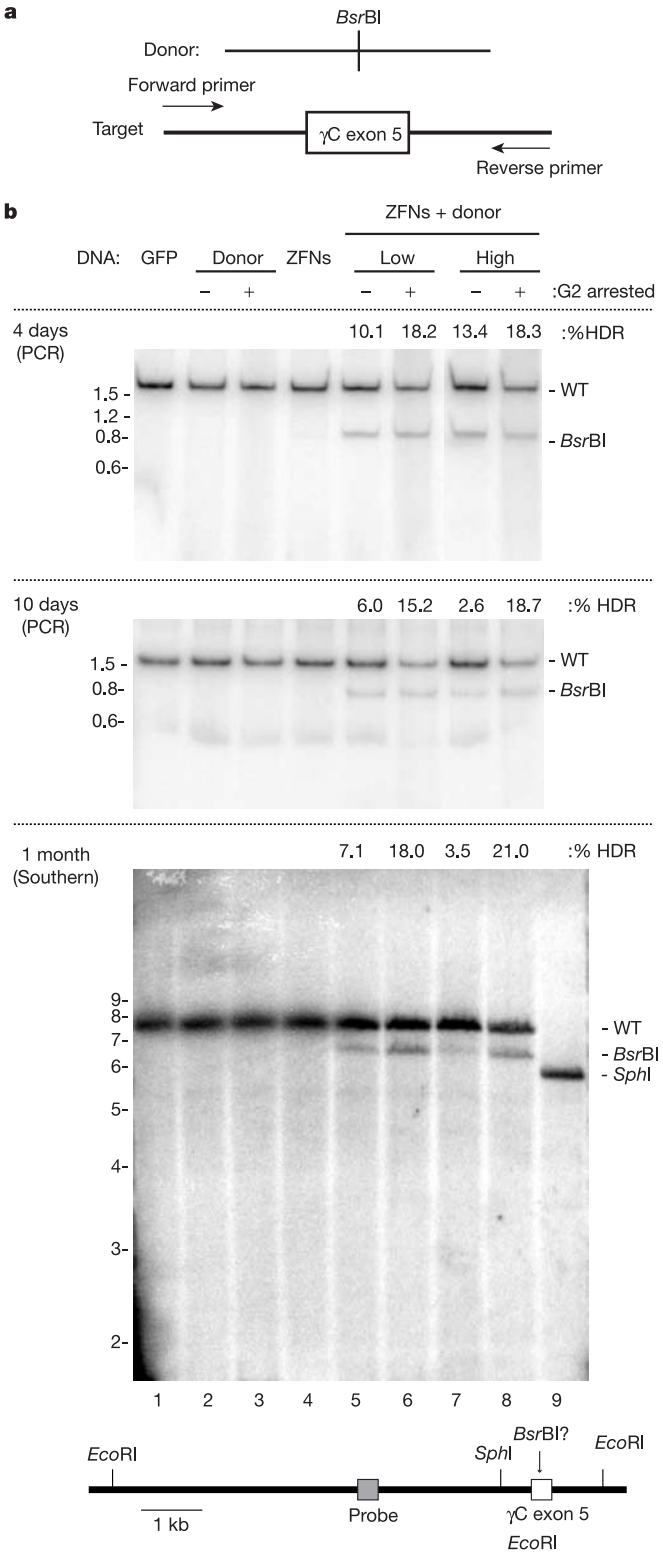

Figure 3 | High-frequency HR at the endogenous *IL2Rγ* locus driven by designed ZFNs. a, Schematic of the donor construct and HR detection scheme. **b**, Cells were transfected with the indicated plasmids at two different concentrations ('low' and 'high'), and HR frequency measured by PCR-amplification of the *IL2Rγ* locus, digestion with *Bsr*BI, PAGE, and autoradiography after 4 (top) and 10 (middle) days. In a separate experiment (bottom), genomic DNA was harvested after 1 month, digested with *Eco*RI and *Bsr*BI and HR frequency measured by Southern blotting. Percentage of chromatids carrying the novel restriction sites is indicated (% homology-directed repair, HDR).

in exon 5 (ref. 30). After initial tests for DNA binding, we optimized the ZFP–DNA interface: analysis of a large archive of designed and *in-vitro*-selected zinc-finger modules guided single amino-acid substitutions in the recognition α-helices to yield ZFPs with improved *in vitro* binding and cleavage properties (data not shown). These optimized ZFNs were ~5 times more potent at reporter gene correction than the original nucleases (Fig. 2b), and were well tolerated by the cells, with comparable numbers of GFP-positive cells observed at 3 and 7 days post-transfection (Fig. 2b).

We then used these optimized ZFNs to modify the sequence at the endogenous *IL2Rγ* locus. We engineered a donor plasmid containing a fragment of the *IL2Rγ* locus to carry a silent point mutation that creates a novel restriction enzyme recognition site in the exon 5 sequence (Fig. 3a). ZFN-induced HR using this donor would introduce this site into the cognate chromosomal location, and the frequency of this event can be accurately (Supplementary Fig. 1) measured by a limited-cycle polymerase chain reaction (PCR)–restriction digest assay (see Methods). Ninety-six hours after transfecting K562 cells with the donor plasmid and ZFNs we observed an HR frequency of 18% (Fig. 3b), and the conversion of the Pro 229 codon to the donor-specified form was confirmed by sequencing the *IL2Rγ* locus (data not shown). In asynchronously growing cells, HR was observed in 10% of the *IL2Rγ* alleles (Fig. 3b).

To determine whether this conversion is stable over time, we analysed genomic DNA isolated from cells at 10 days and at 1 month post-transfection with donor DNA and the ZFNs. We found that 18–21% of *IL2Rγ* loci contained the donor-specified restriction site (Fig. 3b), in both cell samples. These data eliminate the possibility of a systematic PCR-based error in the experiments, because DNA isolated at 1 month was analysed by Southern blotting. Further analysis of these DNA samples by Southern blotting failed to detect ZFN-induced donor plasmid misintegration or gross rearrangements

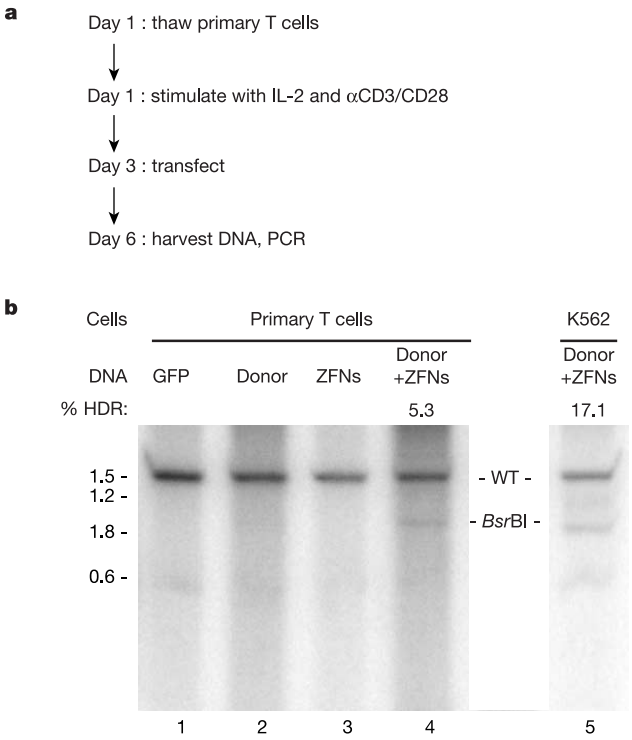

Figure 4 | High-frequency HR at the endogenous *IL2Rγ* locus driven by designed ZFNs in primary human CD4$^+$ T cells. a, Outline of experiment. **b**, Primary human T cells were transfected with the indicated plasmids, and HR frequency measured by the PCR assay described above (a DNA sample from ZFN- and donor-transfected K562 cells was processed in parallel for reference). As gauged by GFP fluorescence, T-cell transfection efficiency in this experiment was 30%.

of the *IL2Rγ* locus (Supplementary Figs 2 and 3). Identical HR frequencies at the endogenous *IL2Rγ* gene were observed in cell samples harvested at 4 days and after 1 month of cell expansion (Fig. 3b, top and bottom panel), that is, no decrease in the number of corrected chromosomes was observed after a 1-month period in culture, in contrast to published work using three-finger ZFNs[26]. To determine whether ZFN-driven targeted genome modification can be carried out in primary cells, we used the same protocol and *IL2Rγ*-targeting ZFNs on human $CD4^+$ T cells, and observed HR at a frequency of 5.3% (Fig. 4b)—that is, comparable to that seen earlier in transformed cells (Fig. 3b) when adjusted for the lower transfection efficiency in T cells (30%). Taken together, these data demonstrate that designed ZFNs can evoke HR to generate permanent, precise modification of the genome in 20% of the chromatids in the absence of selection.

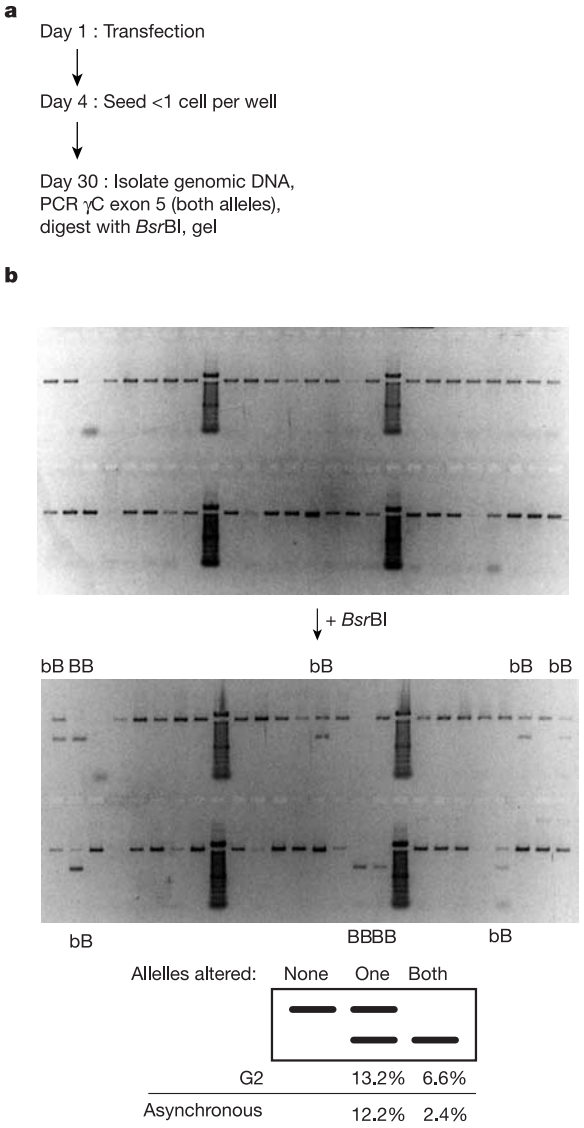

Figure 5 | High-frequency monoallelic and biallelic alteration of *IL2Rγ* driven by designed ZFNs. **a**, Experimental outline. **b**, Fragments of the X chromosome carrying exon 5 of *IL2Rγ* were isolated by PCR from single-cell clones after three weeks of expansion after being transfected with ZFNs and *Bsr*BI-carrying donor DNA and arrested for 30 hours in G2. Forty-eight colonies are shown here of the 96 in total that were genotyped; blank lanes correspond to samples in which DNA isolation failed for technical reasons. Clones were genotyped by digestion with *Bsr*BI (heterozygous, bB; homozygous, BB). The percentage of cells exhibiting each genotype is indicated.

Biallelic gene modification at an endogenous locus

To further characterize the ability of ZFNs to induce HR, we transfected K562 cells with the *Bsr*BI donor plasmid and ZFNs and then performed limiting dilution to isolate single clones. We genotyped exon 5 in each clone by PCR and digestion with *Bsr*BI, and found that 13.2% of the clones had converted a single allele of the *IL2Rγ* gene to the donor-specified form, and that 6.6% were homozygous for that alteration (Fig. 5b). In asynchronously growing cells we found an overall HR frequency of 12.2% and a bi-allelic HR frequency of 2.4% (Supplementary Fig. 4). These data confirm the

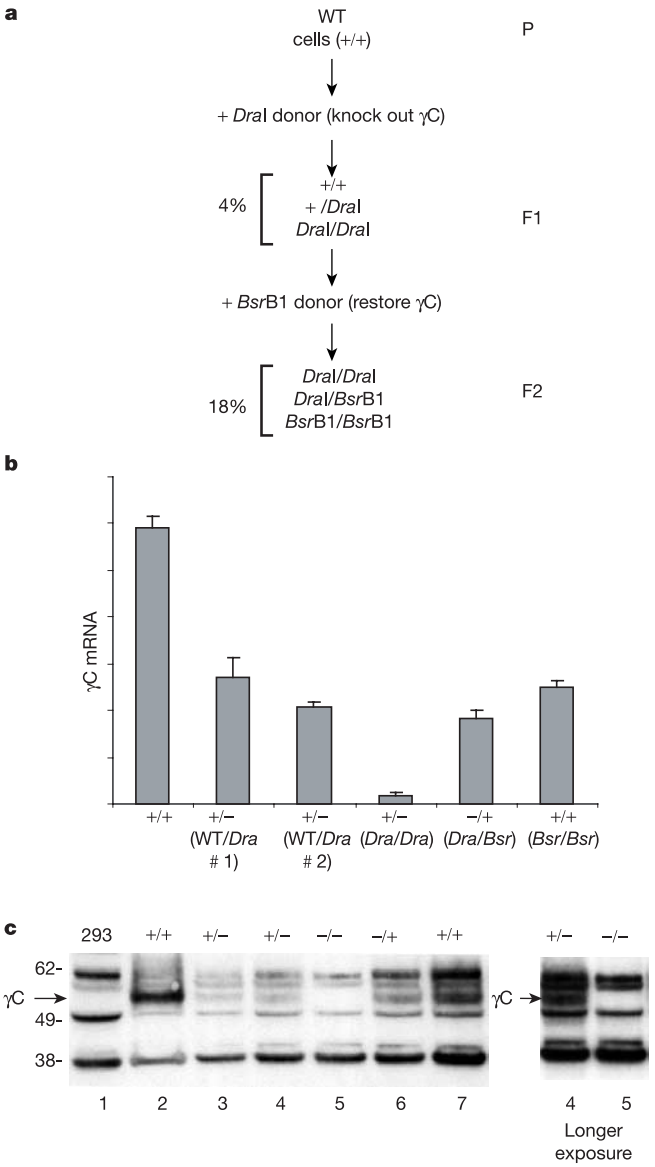

Figure 6 | Serial modification of the *IL2Rγ* locus demonstrates the usefulness of ZFNs in somatic cell genetics and the potential for gene correction therapy. **a**, Experimental outline: *IL2Rγ* is disabled in K562 cells using ZFNs and a donor DNA molecule carrying a frameshift and a novel restriction site (*Dra*I). Cells homozygous for the knockout allele are then reverted to a wild-type phenotype using the same ZFNs and a donor that restores the γC open reading frame in exon 5 and creates a diagnostic restriction site (*Bsr*BI). Cell generations are shown on the right. **b**, **c**, Precise correlation between cell genotype and γC mRNA levels as measured by quantitative real-time RT–PCR (**b**; error bars are the standard deviation in measurement of four samples) and of γC protein detected by western blotting (**c**). Two different heterozygous clones were analysed: WT/*Dra* #1 and #2. HEK 293 cells do not express γC. WT, wild-type.

overall frequencies found by our analysis of cell populations (Fig. 3) and further indicate that ZFNs can be used to rapidly invoke single-step, permanent biallelic modification of a specific locus in the human genome without selection.

ZFN-driven targeted alteration of mRNA and protein levels

We next demonstrated the use of ZFN-mediated HR to alter or correct the expression of an endogenous gene. We created an *IL2Rγ* donor molecule that would introduce a single base pair frameshift concomitant with a *Dra*I recognition site in exon 5. We transfected K562 cells with the *Dra*I donor and ZFN expression plasmids, and isolated cell lines in which one or both alleles had been mutated (Fig. 6). Quantitative reverse trancription (RT)–PCR and western blot analysis showed that the resulting heterozygous cells had reduced amounts of mRNA and protein and that cells homozygous for the *Dra*I site contained no detectable γC protein or mRNA (Fig. 6b, c); the lack of mRNA is probably due to nonsense-mediated decay[31]. We next corrected this induced frameshift mutation by transfecting cells homozygous for the *Dra*I-donor-derived mutation with the *Bsr*BI donor and ZFNs, followed by isolation of cell clones with one or both alleles of *IL2Rγ* corrected. Quantitative RT–PCR and western analysis demonstrated that these cells regained expression of both γC mRNA and protein (Fig. 6c). RT–PCR of similarly passaged wild-type cells confirmed that the partial recovery of γC mRNA levels in homozygous-corrected cells relative to the parental cell line (Fig. 6b) is a consequence of decreased γC expression in the course of passaging these cells (data not shown). These experiments demonstrated that we can efficiently create cell lines carrying defined heterozygous and homozygous alterations in the genome, which lead to changes in cognate mRNA and protein levels. Furthermore, we have shown that ZFNs can efficiently correct a mutation at a locus mutated in human disease.

Discussion

The results presented here establish a general method for the rapid and permanent modification of the human genome at a specific location both in transformed and in primary cells. Building on extensive previous work[15,32,33], we combine high-fidelity DNA recognition by engineered C_2H_2 ZFPs and homology-directed repair of double-strand breaks to achieve HR frequencies that make feasible experimental procedures using human cells that were previously considered impractical. After only a 4-day period and in the absence of any selection for the desired event, ZFNs induce the modification of an endogenous locus in ~20% of the population, with approximately 7% of the cells becoming homozygous for the donor-specified genotype (Figs 3–5). We find these modified cells to be stable for extended periods in cell culture while transcriptionally and translationally manifesting their new genotype (Fig. 6).

The practical application of ZFN-driven gene correction in clinical settings or in basic research requires the ability to invoke a DSB at an unmodified endogenous locus with high efficiency. Work over the past 15 years has evolved the C_2H_2 class of zinc-finger DNA-binding domains into the only engineered peptide motif capable of binding to essentially any DNA sequence[10,13,34], and modulating the function of specific genes *in vivo*[12,15]. A large archive of zinc-finger modules has been generated using both rational design and selection methods to provide the technical platform necessary for targeting endogenous loci[14,15]. In agreement with the results of published work[25,26,35] we find that both affinity and specificity are critical determinants for ZFN efficacy in driving selection-free gene correction, and of the long-term stability of the modified cell (Figs 2 and 3); for the present work, we used a large archive of both designed and selected zinc-finger modules to guide the ZFN optimization effort.

ZFNs can be used to rapidly generate cells heterozygous or homozygous for a genotype of interest. The frequency and precision of ZFN-driven HR revolutionizes the existing toolbox for mammalian somatic cell genetics and the study of gene function both in basic research and in drug discovery efforts. Our ultimate goal is to apply the ZFN technology to the therapeutic modification of human cells in the clinic. This application will require extensive study of ZFN safety in appropriate model systems. Limitations are imposed by the need to deliver ZFN-encoding and donor DNA molecules to cells, and the potential immunogenicity of the ZFNs. These concerns are lessened in therapeutic strategies involving *ex vivo* autologous cell manipulation and monogenic diseases of haematopoiesis, including immune deficiencies and haemoglobinopathies, which lend themselves to this approach because both the appropriate target cells and the delivery methods have been extensively studied[3,4]. Importantly, the 'hit and run' mechanism of ZFN action uncouples the therapeutically beneficial changes made to the genome from any need to integrate exogenous DNA, while still generating a permanently modified cell.

METHODS

ZFN and donor construct assembly. Zinc-finger proteins were designed against the coding sequence of eGFP and assembled exactly as described[36,37], to yield the following ZFP moieties (target gene; ZFP name; target sequence; recognition α-helices): eGFP; ZFP-L; GGGGTAGCG; RSDDLTR, QSGALAR, RSDHLSR and eGFP; ZFP-R; GAAGCAGCA; QSGSLTR, QSGDLTR, QSGNLAR. Zinc-finger proteins for targeting the *IL2Rγ* locus were assembled from an archive of *in-vitro*-selected modules[29,38], assembled as described[32,39], and after α-helix optimization, yielded the following ZFP moieties: IL2Rγ; ZFP-R*; ACTCTGTGGAAG; RSDNLSV, RNAHRIN, RSDTLSE, ARSTRTN and IL2Rγ; ZFP-L*; AAAGCGGCTCCG; RSDTLSE, ARSTRTT, RSDSLSK, QRSNLKV. Assembled ZFPs were cloned in-frame as NH_2-terminal fusions to the catalytic domain of *Fok*I[17,25,26] into pcDNA 3.1 (Invitrogen). The donor plasmids for correcting the defective eGFP gene (see below) and for modifying the endogenous γC locus are described in the Supplementary Information.

Gene correction in tissue culture cells. A defective eGFP reporter gene with nucleotides 229–236 relative to the start codon replaced with a stop codon and a 2-bp frameshift in the open reading frame was generated by standard PCR mutagenesis techniques, cloned into pcDNA4/TO vector (Invitrogen) and stably introduced into HEK 293 T-Rex cells (Invitrogen). A cell line with a single copy of the plasmid integrated into the genome was identified and used for all experiments. A reporter cell line carrying GFP disabled with an insertion of the ZFN-targeted stretch of *IL2Rγ* was made as described[26]; the absolute HR frequency observed when using this reporter line is lower than that seen on endogenous loci, most probably owing to the interruption of the donor–target alignment with an exogenous DNA stretch. HEK 293 T-Rex (Invitrogen) and K562 (ATCC) cells were grown according to the suppliers' instructions and transfected with LipofectAMINE 2000 (Invitrogen) or by Nucleofection (Solution V, Program T16) (Amaxa Biosystems) according to the manufacturer's protocol (see Supplementary Information). Gene correction of the mutated GFP reporter was measured by FACS. For analysis of gene correction at the γC locus, genomic DNA was amplified in 20 cycles of PCR with primers that hybridize to the chromosomal *IL-2Rγ* locus immediately outside of the region corresponding to the 1.5-kilobase (kb) donor sequence, and analysed by digestion with *Bsr*BI, gel electrophoresis and autoradiography as described in Supplementary Information. Southern blotting (Fig. 3) was performed by probing *Eco*RI-, *Bsr*BI- and *Dpn*I-digested genomic DNA transferred to Nytran Plus (Schleicher and Schuell) with a 340-bp fragment of the *IL2Rγ* locus labelled with α-^{32}P-dCTP and α-^{32}P-dATP (Roche) in RapidHyb buffer (Amersham Pharmacia). Quantitative RT–PCR and western blot assays for γC were performed following standard procedures described in Supplementary Information.

Human $CD4^+$ T cells (AllCells) were grown according to the supplier's instructions. Cells were activated using 20 Units per ml IL-2 (R&D Systems) and αCD3/CD28 beads (Miltenyi Biotec, T Cell Activation/Expansion kit), and transfected with ZFN and donor constructs at an efficiency of ~30%, using an Amaxa nucleofector according to the manufacturer's instructions. DNA was isolated and HR at exon 5 of *IL2Rγ* was measured by PCR as above, except that 25 cycles of PCR were performed.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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