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Raman Bahal

Yale University

Et al.

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In vivo correction of anaemia in β-thalassemic mice by γPNA-mediated gene editing with nanoparticle delivery

Raman Bahal1,*, Nicole Ali McNeer1,2,*, Elias Quijano2, Yanfeng Liu1, Parker Sulkowski1,3, Audrey Turchick1,3, Yi-Chien Lu4, Dinesh C. Bhunia5, Arunava Manna5, Dale L. Greiner6, Michael A. Brehm6, Christopher J. Cheng2, Francesc López-Giráldez7, Adele Ricciardi1,2, Jagadish Beloor8, Diane S. Krause4, Priti Kumar8, Patrick G. Gallagher9, Demetrios T. Braddock10, W. Mark Saltzman2, Danith H. Ly5 & Peter M. Glazer1,3

The blood disorder, β-thalassaemia, is considered an attractive target for gene correction. Site-specific triplex formation has been shown to induce DNA repair and thereby catalyse genome editing. Here we report that triplex-forming peptide nucleic acids (PNAs) substituted at the γ position plus stimulation of the stem cell factor (SCF)/c-Kit pathway yielded high levels of gene editing in haematopoietic stem cells (HSCs) in a mouse model of human β-thalassaemia. Injection of thalassemic mice with SCF plus nanoparticles containing γPNAs and donor DNAs ameliorated the disease phenotype, with sustained elevation of blood haemoglobin levels into the normal range, reduced reticulocytosis, reversal of splenomegaly and up to 7% β-globin gene correction in HSCs, with extremely low off-target effects. The combination of nanoparticle delivery, next generation γPNAs and SCF treatment may offer a minimally invasive treatment for genetic disorders of the blood that can be achieved safely and simply by intravenous administration.
Gene editing in haematopoietic stem cells (HSCs) may provide a strategy for treatment of inherited disorders such as sickle cell disease and β-thalassaemia. Methods for gene editing include targeted nucleases such as zinc-finger nucleases and CRISPR/Cas9 (ref. 2), short fragment homologous recombination and triplex-forming oligonucleotides. Recent excitement has focused on CRISPR/Cas9 technology because of facile reagent design. However, like zinc-finger nucleases, the CRISPR approach introduces an active nuclease into cells, which can lead to off-target cleavage in the genome. As an alternative, we have pursued triplex-forming peptide nucleic acids (PNAs) designed to bind site-specifically to genomic DNA via strand invasion and formation of PNA/DNA/PNA triplexes (via both Watson–Crick and Hoogsteen binding) with a displaced DNA strand. PNAs consist of a charge-neutral peptide-like backbone and nucleobases enabling hybridization with DNA with high affinity. PNA/DNA/PNA triplexes recruit the cell’s endogenous DNA repair systems to initiate site-specific modification of the genome when single-stranded ‘donor DNAs’ are co-delivered as templates containing the desired sequence modification.

Our prior work has suggested that PNA-induced genome modification is mediated by the nucleotide excision repair and homology-dependent repair (HDR) pathways. Both nucleotide excision repair and HDR are high fidelity pathways, and the PNAs lack any intrinsic nuclease activity; together these features may account for the very low frequencies of off-target genotoxicity seen with PNA-mediated gene editing compared with nuclease based approaches. We have also recently shown that tail-clamp PNAs (tcPNAs) with an extended Watson–Crick binding domain can enhance gene editing in human haematopoietic cells with increased efficiency and specificity and that polymer nanoparticles (NPs) can effectively deliver these molecules into human HSCs both ex vivo and in vivo in a humanized mouse model.

Here, we use next generation triplex-forming γPNAs containing a polyethylene glycol substitution at the gamma position for enhanced DNA binding. Delivered via polymer NPs, the γPNAs mediate increased gene editing in HSCs both ex vivo and in vivo in two different mouse models, one carrying a β-globin/green fluorescent protein (GFP) fusion transgene and the other carrying the human β-globin gene replacing the mouse β-globin genes and containing a β-thalassaemia-associated splicing mutation at IVS2-654 (refs 17,18). We find that treatment with stem cell factor (SCF), the k-Kit ligand, further enhances PNA-mediated gene editing, an effect associated with increased DNA repair. Treatment of thalasssemic mice with NPs containing γPNAs and donor DNAs, plus SCF, produced up to 7% gene editing in HSCs, with elevation of blood haemoglobin levels for 140 days post-treatment, reduction in reticulocyte counts and reversal of splenomegaly. γPNA treatment also yielded gene editing in human CD34+ HSCs ex vivo at a frequency of 5% with a single treatment, with very low off-target effects. These results indicate that chemical advances in PNA design and in vivo delivery via polymer NPs can mediate clinically relevant levels of gene editing. They also identify SCF treatment as a potential pharmacologic strategy to increase gene editing that may be applicable not only to triplex-forming PNAs but also to approaches such as CRISPR/Cas9.

Results

Triplex-forming PNA design for gene editing. To quantitatively assay for gene editing, we used a mouse model with a β-globin/GFP fusion transgene consisting of human β-globin intron 2 carrying a thalassaemia-associated IVS2-654 (C→T) mutation embedded within the GFP coding sequence, resulting in incorrect splicing of β-globin/GFP mRNA and lack of GFP expression. PNA-mediated triplex-formation induces recombination of the genomic site with a 60-nucleotide sense donor DNA homologous to the β-globin intron 2 sequence except for providing a wild-type nucleotide at the IVS2-654 position. Correction of the splice-site mutation yields expression of functional GFP (Fig. 1a) providing a phenotypic read-out of editing quantifiable by flow cytometry.

We designed a series of tcPNAs to bind to polyuridine stretches in the β-globin intron near the IVS2-654 mutation (Fig. 1b). One of the tcPNAs and a scrambled sequence control were synthesized to contain partial substitution with a mini-polyethylene-glycol group at the γ position (tcPNA; Fig. 1c,d) within their Watson–Crick binding domains. We made the substitutions in the Watson–Crick domains because in prior work γPNAs have been shown to enhance strand invasion and DNA binding in the Watson–Crick binding mode due to helical pre-organization enforced by the modification. We hypothesized that this would enhance the binding of the tcPNAs because strand invasion and Watson–Crick DNA/DNA duplex formation is an important component in the formation of PNA/DNA/PNA triplexes. Partial substitution was performed because it is sufficient to improve binding affinity and to confer helical pre-organization.

γtcPNA4 matches the sequence of tcPNA1 except that it contains γ units at alternating positions in the Watson–Crick domain (Fig. 1d). Scrambled γtcPNA (γtcPNA4-Scr) has the same base composition as γtcPNA4 but a scrambled sequence. All tcPNA oligomers were synthesized with 3 lysines at both termini to improve solubility and increase binding affinity (Fig. 1d). Purification and characterization of the synthesized PNAs were performed by high-performance liquid chromatography analyses and matrix-assisted laser desorption/ionization time-of-flight, respectively (Supplementary Fig. 1 and Supplementary Table 1). Gel shift assays to assess the binding of the tcPNAs to DNAs containing the respective target sequences showed that all bound specifically to their target sites (Supplementary Fig. 2A). No binding was seen in the case of the scrambled sequence γtcPNA4-Scr.

In prior work, we showed that poly(lactic-co-glycolic acid) (PLGA) NPs can effectively deliver PNA/donor DNA combinations into primary human and mouse haematopoietic cells with essentially no toxicity. Here, tcPNAs and donor DNAs, at a molar ratio of 2:1, were incorporated into poly(lactic-co-glycolic acid) (PLGA) NPs. The NP formulations were evaluated by scanning electron microscopy and dynamic light scattering. All the NPs exhibited sizes within the expected range (Fig. 1e and Supplementary Fig. 2B,C) and showed uniform charge distribution (Supplementary Fig. 2D). Nucleic acid release profiles showed that γ modifications did not impair release from NPs (Supplementary Fig. 2E).

Ex vivo gene editing in bone marrow cells. Bone marrow (BM) cells harvested from β-globin/GFP mice were treated ex vivo with PLGA NPs containing tcPNA1/donor DNA, tcPNA2/donor DNA and tcPNA3/donor DNA. After 48 h, the percentage of GFP (+) cells was quantified via flow cytometry (Fig. 1f). The higher gene editing activity of tcPNA1 is likely due to its longer Hoogsteen binding domain, an effect that we have previously observed. NPs containing the γ-substituted tcPNA (γtcPNA4) and donor DNA yielded significantly higher gene modification (1.62%; Fig. 1g and Supplementary Fig. 3A), showing that the MPγ substitutions confers increased biological activity that correlates with their improved binding properties. NPs with the γtcPNA4-Scr produced no modification (Fig. 1g).
Figure 1 | Gene editing using γPNAs in mouse bone marrow. (a) Strategy for targeted correction of a β-globin gene IVS2-654 (C→T) mutation in β-globin/GFP transgenic mice using triplex-forming tail-clamp PNAs and donor DNAs. (b) tcPNA and γtcPNAs designed to bind to homopurine regions within intron 2 of the human β-globin gene near IVS2-654 (C→T). (c) DNA, unmodified PNA and MPγPNA. (d) tcPNAs and γtcPNAs to bind to the positions indicated in B. γtcPNA4-Scr is a scrambled version of γtcPNA4. Bold/underline indicates γPNA residues. K indicates lysine; J, pseudoisocytosine (for c) for pH-independent triplex formation. O, 8-amino-2,6,10-trioxaoctanoic acid linkers connecting the Hoogsteen and Watson–Crick domains of the tcPNAs. (e) Scanning electron microscope images of nanoparticles. Scale bar, 2.0 μm. (f) Gene correction of the IVS2-654 (C→T) mutation within the β-globin/GFP fusion gene in mouse BM cells treated ex vivo with NPs containing the indicated tcPNAs and donor DNA. %GFP + was determined by flow cytometry and indicates successful gene editing. Data are mean ± s.e., n = 3; statistical analysis by Student’s t-test, *P < 0.005. (g) %GFP + cells in mouse BM after ex vivo treatment with NPs containing tcPNA1, γtcPNA4 or γtcPNA4-Scr, plus donor DNAs. Data are mean ± s.e.m., n = 3; analysis by Student’s t-test, *P < 0.05. (h,i) Quantification of γH2AX foci by immune fluorescence microscopy, indicative of DNA DSBs in primary fibroblasts (from the β-globin/GFP transgenic mice) either untreated or treated with 5 Gy of IR, blank NPs, NPs containing γtcPNA4 and donor DNA, lipofectamine alone, lipofectamine transfection of a Cas9 expression vector, lipofectamine transfection of a Cas9 and a separate guide RNA expression vector (targeting the same site in β-globin sequences as γtcPNA4; Cas9 + gRNA), or transfection of a vector containing both Cas9 and guide RNA (Cas9 and gRNA). Quantification as per cent of cells with 15 or more foci (h) or average number of foci per cell (i). 100 cells were counted per condition, data are mean ± s.e.m., n = 3; analysis by Student’s t-test, *P < 0.05.
BM cells treated with either blank NPs or NPs containing \(\gamma\)tcPNA4/donor DNA were plated in methylcellulose medium supplemented with cytokines for growth of granulocyte/macrophage colonies (colony-forming unit (CFU)-G, CFU-M and CFU-GM) or combined colonies (CFU-GEMM, granulocyte, erythroid, monocye/macrophage and megakaryocyte). The two sets of treated cells formed myeloid and erythroid colonies at similar frequencies (Supplementary Fig. 3B,C), suggesting that treatment with \(\gamma\)tcPNA4 and donor DNA does not impair the ability of the progenitor cells to proliferate and differentiate. Sequencing analysis of genomic DNA from selected GFP-positive methylcellulose colonies confirmed the presence of the targeted gene modification in the \(\beta\)-globin/GFP transgene at the IVS2-654 base pair (Supplementary Fig. 3D). Also, there was no induction of the inflammatory cytokines in the treated BM cells (Supplementary Fig. 3E), consistent with prior work with NPs containing standard PNA\(_2\)12,14,21.

In assays for genotoxicity, there was no detectable increase in DNA double-strand breaks (DSBs) in the BM cells treated with \(\gamma\)tcPNA4/donor DNA-containing NPs compared with blank NPs based on a single-cell gel electrophoresis assay (Comet assay; Supplementary Fig. 4A). We also assayed for DSBs by measuring the production of \(\gamma\)H2AX foci. \(\gamma\)H2AX foci are detected in nuclei by immune fluorescence and document a chromatin modification that occurs upon DSB formation via DNA damage response signalling22. Treatment of the BM cells with NPs containing \(\gamma\)tcPNA4/donor DNA did not produce any \(\gamma\)H2AX foci above the low background that is seen in untreated cells (assayed by flow cytometry as shown in Supplementary Fig. 4B). Treatment of the cells with 5 Gy of ionizing radiation (IR) was included as a positive control for induction of DSBs. We also used primary fibroblasts from the mice for this assay, since adherent cells facilitate more robust quantification of foci by immune fluorescence microscopy. Treatment of the fibroblasts with NPs containing \(\gamma\)tcPNA4/donor DNA again did not produce \(\gamma\)H2AX foci above the low background in untreated or blank NP treated cells (quantified in Fig. 1h as per cent of cells with more than 15 foci and in Fig. 1i as average number of foci per nucleus, with corresponding immune fluorescence images of foci shown in Supplementary Fig. 5). For comparison, transfection of a vector expressing the Cas9 nuclease yielded an increase in \(\gamma\)H2AX foci (Fig. 1h,i). Co-expression of a guide RNA designed to bind to the same sequence in the \(\beta\)-globin gene as \(\gamma\)tcPNA4 (either via the same plasmid or via a plasmid separate from the Cas9 vector) reduced the number of induced DSBs, but to a level that was still above background (Fig. 1h,i and Supplementary Fig. 5).

Elevated gene editing by \(\gamma\)tcPNAs in CD117\(^+\) cells. Previous work suggested that there might be increased PNA-mediated editing in colony-forming progenitors12. To test this, we treated whole BM cells with either blank NPs, NPs containing tcPNA1/donor DNA, or NPs containing \(\gamma\)tcPNA4/donor DNA. Two days later, flow cytometry was performed to assess the frequency of GFP\(^+\) cells within selected sub-populations. CD117\(^+\) cells showed elevated gene editing compared with the total CD45\(^+\) cell population (Fig. 2a), with 8.6% in CD117\(^+\) cells after a single treatment with \(\gamma\)tcPNA4/donor DNA NPs. The less potent tcPNA1/donor DNA NPs still yielded an elevated correction frequency of 2.1% in CD117\(^+\) cells compared with the total BM (although fourfold lower than \(\gamma\)tcPNA4). Next, we sorted for CD117\(^+\) cells before treatment with the NPs (Fig. 2b). An elevated percentage of modification (7.2%) was again seen after a single treatment (Fig. 2b).

The c-Kit pathway mediates increased gene editing. CD117 (also known as mast/stem cell growth factor receptor or proto-oncogene c-Kit protein) is a receptor tyrosine kinase expressed on the surface of haematopoietic stem and progenitor cells and other cell types. SCF, the ligand for c-Kit, causes dimerization of the receptor, activating its kinase activity to trigger signalling pathways that impact survival, proliferation and differentiation.

We asked whether c-Kit-dependent signalling is required for elevated gene correction or whether CD117 simply serves as a marker for the increased susceptibility. First, we tested for gene editing in pre-sorted CD117\(^+\) cells treated with or without the c-Kit ligand, SCF, and we observed a significant increase in \(\gamma\)tcPNA4/donor DNA-mediated gene editing (up to almost 15%) in the SCF-treated cells (Fig. 2c and Supplementary Fig. 6). We next assayed for \(\gamma\)tcPNA4/donor DNA NP-mediated gene editing in pre-sorted CD117\(^+\) cells in the presence or absence of kinase inhibitors (Fig. 2d). Dasatinib, which inhibits the c-Kit kinase in addition to the BCR/Abl and Src kinases, reduced the gene editing from 7 to 2.0%. Inhibitors of factors downstream of c-Kit, including mitogen/extracellular signal-regulated kinase (MEK; Binimetinib; MEK162) and phosphatidylinositol-3-kinase (BKM120), also decreased gene editing in CD117\(^+\) cells to 2.6 and 4.1%, respectively (Fig. 2d).

We also tested tcPNA1 (no gamma substitutions) for gene editing in pre-sorted CD117 cells treated or not with SCF. We found that SCF boosted tcPNA1/donor DNA gene editing up to 5% versus about 1.5% without SCF (Supplementary Fig. 7). While substantial, this was threefold lower than the almost 15% frequency seen with the \(\gamma\)tcPNA4/donor DNA NPs in conjunction with SCF treatment of the pre-sorted CD117 cells (Fig. 2c). Overall, these results indicate that the SCF/c-Kit signalling can enhance gene editing and identify SCF as a potential agent to stimulate PNA-mediated gene modification. In addition, the results further show that \(\gamma\)tcPNAs provide increased gene editing potential as compared with regular tcPNAs.

Increased DNA repair gene expression upon activation of c-Kit. To explain the increased gene editing in CD117 cells, we tested whether CD117 might be associated with differential uptake of the NPs. However, there were no differences in uptake across several BM cell sub-populations (Supplementary Fig. 8). Next, we examined gene expression patterns in the c-Kit\(^+\) cells to test the hypothesis that there might be increased DNA repair gene expression. RNA from sorted CD117\(^+\) and CD117\(^-\) cells from the \(\beta\)-globin/GFP mice was analysed using Illumina arrays (Supplementary Fig. 9A–C). We found that numerous DNA repair genes, including BRCA1, BRCA2, Rad51, ERCC2, XRCC2 and XRCC3, showed higher levels of expression in CD117\(^+\) cells (Supplementary Figs 10 and 11a). The elevated expression two genes likely to play a role in PNA-induced recombination, BRCA2 and Rad51, was confirmed in CD117\(^+\) cells by quantitative PCR with reverse transcription (Supplementary Fig. 11B,C) and by western blot (Supplementary Fig. 11D). We went on to test whether SCF treatment could further increase DNA repair gene expression. Gene expression profiling showed additional up-regulation of numerous DNA repair genes by SCF treatment (Fig. 2e and Supplementary Fig. 12A,B).

Because SCF, as a stimulatory cytokine, would be expected to impact the cell cycle in c-Kit\(^+\) cells and because HDR gene expression is known to be elevated in S-phase, we also measured the effect of SCF on cell cycle phase distribution in the pre-sorted CD117 cells. We found that there was a 9% increase in the per cent of cells in S-phase at 48 h after SCF treatment as compared with no SCF treatment (Supplementary Fig. 13). Hence, some
The c-Kit pathway induces functionally elevated DNA repair.

To test whether the above increases in DNA repair gene expression could be correlated with functional differences in DNA repair, we used a luciferase-based assay to quantify repair of DNA DSBs by HDR. In this assay, repair of a DSB in a reporter plasmid via intramolecular homologous recombination creates (‘reactivates’) a functional luciferase gene (Fig. 2f), and so the assay provides a measure of HDR capacity (validation of the assay component of the SCF effect on DNA repair genes could be an indirect effect of cell cycle changes.

**Figure 2** | The SCF/c-Kit + pathway promotes gene editing and DNA repair. (a) %GFP expression in treated mouse BM cells expressing the indicated cell surface markers. Total BM was treated with NPs containing either tcPNA1/donor DNA or γtcPNA4/donor DNA, cells were stained using antibodies specific for the indicated markers and assayed by flow cytometry for marker and GFP expression. Data are mean ± s.e.m, n = 3; statistical analysis by Student’s t-test, *P < 0.05. (b) %GFP expression in pre-sorted CD117 (c-Kit +) cells treated with either NPs carrying γtcPNAs and donor DNAs or blank NPs. Data are mean ± s.e.m, n = 3; statistical analysis by Student’s t-test, **P < 0.005. (c) %GFP expression in pre-sorted CD117 + cells treated with NPs containing γtcPNA4/donor DNA with or without prior treatment with the c-Kit ligand, SCF. Data are mean ± s.e.m, n = 3; statistical analysis by Student’s t-test, *P < 0.05. (d) %GFP expression in pre-sorted CD117 + cells treated with NPs containing γtcPNA4/donor DNA in the presence or absence of c-Kit pathway kinase inhibitors: dasatinib (inhibits c-Kit), MEK162 (inhibits MEK, MEK) and BKM120 (inhibits phosphatidylinositol-3-kinase). Data are mean ± s.e.m, n = 3; statistical analysis by Student’s t-test, *P < 0.05. (e) Heat map showing upregulated genes involved in DNA repair in CD117 + cells with or without SCF treatment; rows are clustered by Euclidean distance measure. (f) Reporter gene assay for HDR activity in CD117 + cells in the presence or absence of c-Kit pathway kinase inhibitors: dasatinib, MEK162 and BKM120 (as above). Inset shows diagram of the luciferase reporter assay for repair of a nuclease-induced DSB by HDR. Luciferase expression occurs only after homologous recombination and is scored as % reactivation of the DSB-damaged plasmid, normalized to a transfection control. Data are mean ± s.e.m, n = 3; statistical analysis by Student’s t-test, ***P < 0.005. (g) HDR assay in CD117 + cells with or without the addition of SCF. Data are shown as mean ± s.e.m, n = 3; statistical analysis by Student’s t-test, **P < 0.005 and ****P < 0.0005.
is shown in Supplementary Fig. 14). The results show increased HDR in CD117+ compared with CD117− cells (Fig. 2f). HDR in CD117+ cells was diminished by the kinase inhibitors MEK162, BKMI20 and dasatinib (Fig. 2g); conversely, it was boosted by SCF treatment (Fig. 2g). These results indicate that c-Kit signalling increases HDR.

In vivo gene editing by PNA/DNA NPs given intravenously.

We next tested the potential for in vivo gene editing in the β-globin/GFP transgenic mice by simple intravenous injection of NPs containing tcPNA1/donor DNA or γtcPNA4/donor DNA, and we further asked whether the editing could be enhanced by SCF treatment. Mice were treated with a single dose of 4 mg NPs, and 2 days later we analysed gene editing in BM and spleen. Some mice also received murine SCF (220 μg kg−1) given by intraperitoneal injection 3 h before the NP injection (Fig. 3a,b, with experimental scheme shown in Supplementary Fig. 15A).

In vivo gene editing was scored by GFP expression in marker-sorted cell populations from BM and spleen (Fig. 3a,b). We observed an average of ~0.2% gene editing in the CD117 BM cells from tcPNA1/donor DNA and SCF-treated mice (Fig. 3a).

Two to threefold higher levels of gene editing were seen in CD117+ cells from BM of the γtcPNA4/donor DNA and SCF-treated mice, with frequencies in the range of 1% in several mice, and average frequencies in the 0.5% range. Similar frequencies were seen in spleen (Fig. 3b).

We confirmed these results by performing deep sequencing analysis on DNA from CD117+ cells isolated from BM and spleen of treated mice (Fig. 3c and Supplementary Fig. 15A), revealing gene editing frequencies in the range of 0.2% in the BM of mice treated with γtcPNA4/donor DNA NPs without SCF and 0.6% in mice receiving SCF along with the γtcPNA4/donor DNA NPs (in a single treatment in each case), consistent with the frequencies of gene correction quantified by GFP expression. Deep sequencing was also used to assess off-target effects in the BM cells of the mice that were treated with SCF and γtcPNA4 and donor DNA NPs (Table 1). By BLAST analysis, we identified seven off-target sites with partial homology to the target site of γtcPNA4 in β-globin intron 2. Extremely low frequencies of off-target effects were found in the γtcPNA4/donor DNA treated mice, with six sites showing no detectable sequence changes out of millions of reads and two sites showing modification frequencies of only 0.0074 and 0.00018% compared with 0.56% at the targeted β-globin site (Table 1). The overall off-target modification frequency at all seven sites combined was 0.00034%, 1,647-fold lower than the frequency of the targeted gene editing.

We also performed cytokine array analyses on plasma derived from mice 48 h after treatment with γtcPNA4/donor DNA NPs. There were no detectable increases in levels of any of the cytokines measured compared with untreated mice (Fig. 3d), with lipopolysaccharide treatment as a positive control showing significantly higher levels of multiple cytokines that were off the scale of the graph (Supplementary Fig. 16). In a separate experiment, mice were treated with γtcPNA4/donor DNA NPs on day 1 and again 3 months later, followed by cytokine analysis of peripheral blood, again showing minimal immune or inflammatory response (Supplementary Fig. 17A,B).

In vivo correction of a β-thalassaemia mutation in mice.

We next tested for correction of a human β-thalassaemia mutation in a mouse disease model, using a transgenic mouse line in which the two (cis) murine adult β-globin genes were replaced with a single copy of the human β-globin gene with the same thalassaemia-associated IVS2-654 mutation as above14. Homozygous mice do not survive, and heterozygotes have a moderate form of β-thalassaemia, with haemolytic anaemia, microcytosis and other erythrocyte morphologies reflecting reduced amounts of mouse β-globin and no human β-globin15,16, consistent with β-thalassaemia. Treatment groups included (1) blank NPs; (2) SCF alone (no NPs); (3) SCF plus γtcPNA4/donor DNA NPs; and (4) SCF plus γtcPNA4-Scr/donor DNA (experimental scheme is shown in Supplementary Fig. 15B). We conducted two otherwise identical replicate experiments except that, in one, the complete blood count (CBC) analyses were continued for 75 days after the last treatment, and in the other for 140 days. In both cases, each treatment group consisted of six mice, and each mouse received four treatments at 2 day intervals at the beginning of the experiment as indicated in Supplementary Fig. 15B. Some animals were maintained long-term for serial CBC analyses; others were killed at intermediate time points for analysis of spleen size and architecture and for deep sequencing of BM-derived cells.

Blood smears at day 0 (before treatment) and at day 36 after the last treatment (Fig. 4a) showed marked improvement in RBC morphology on day 36 in the γtcPNA4/donor DNA treated mice but not in the mice treated with either blank NPs, SCF alone or SCF plus γtcPNA4-Scr/donor DNA. CBC analyses performed on blood samples taken at 30, 45, 60, 75, 90 and 140 days post-treatment from mice in each group showed persistent correction of the anaemia based on blood haemoglobin levels in the mice treated with SCF plus the γtcPNA4/donor DNA NPs (Fig. 4b), with elevation of the blood haemoglobin levels into the normal range. The anaemia was not improved in any of the controls. We also observed reduced reticulocyte counts in mice treated with SCF plus the γtcPNA4/donor DNA NPs but not in the mice treated with blank NPs (Fig. 4c). In addition, the γtcPNA4/donor DNA treated mice also showed reduced splenomegaly at 36 days post-treatment (Fig. 4d).

Consistent with reduced splenomegaly, histologic examination of spleens from mice on days 36 and 75 showed substantially improved splenic architecture specifically in the γtcPNA4/donor DNA treated mice (Fig. 4e and Supplementary Figs 18 and 19). The regular splenic pattern of white pulp (lymphoid follicles) surrounded by rims of red pulp is disrupted in the β-thalassemic animals due to extramedullary haematopoiesis, causing an expansion in red pulp (causing the splenomegaly) and disruption of the white pulp. The CD61 and Ecad immunohistochemical stains (Supplementary Fig. 18) highlight the increased cellularity characteristic of extramedullary haematopoiesis and demonstrate that the expanded red pulp includes elevated megakaryocytes and erythroid precursors, respectively. This increased cellularity is substantially ameliorated in the γtcPNA4/donor DNA treated mice (Supplementary Fig. 18).

Deep sequencing analyses were performed on total BM cells of mice on day 36 post-treatment. Correction of the targeted mutation was seen at a frequency of almost 4% in the γtcPNA4/donor DNA treated group (Fig. 5a; combined analysis of 3 mice), whereas no correction was seen in the mice treated with blank NPs (Fig. 5a). Deep sequencing was also used to assess off-target effects in the BM cells at seven sites with partial homology to the binding site of γtcPNA4 in the β-globin gene. We found extremely low frequencies of off-target effects in the γtcPNA4/donor DNA-treated thalassemic mice (Table 2). The overall off-target modification frequency was 0.0032%, 1,218-fold lower than the frequency of β-globin gene editing.

In addition, we sorted the cells from the BM of the SCF and γtcPNA4/donor DNA NP treated mice (the BM was collected 65 days after the last treatment) for markers consistent with several stem/progenitor cell populations23, and we again performed deep sequencing of the β-globin gene. This revealed evidence for gene editing at a frequency of 6.9% in Lin-Sca1+ cKit+ CD150+...
CD135-cells (Fig. 5a and Supplementary Fig. 20), a population that is highly enriched for long-term HSCs. We also observed gene editing in multiple other progenitor populations (Fig. 5a). We also noted that the 

\[ \beta \]-thalassemic animals had elevated levels of CD117\(^{+}\) cells in their BM compared with the phenotypically normal 

\[ \beta \]-globin/GFP transgenic mice (Supplementary Fig. 21), indicative of the stress erythropoiesis in these animals. We also found that the proportion of CD117\(^{+}\) cells was further increased by SCF treatment. These elevated levels of CD117\(^{+}\) cells, and the stress erythropoiesis that underlies them, could explain, in part, the increased susceptibility to gene editing in the thalassemic mice.

**Gene editing by \( \gamma \)tcPNAs in CD34\(^{+}\) human HSCs.** We next tested the gene editing potential of \( \gamma \)tcPNA4/donor DNA NPs in...
human CD34+ cells. Because we did not have cells from a thalassaemic patient, we obtained human CD34+ cells from healthy donors from a cell bank. Consequently, we used a modified donor DNA designed to introduce a mutation at position IVS2-654 rather than correct it. One day after the CD34+ cells were thawed into stem cell medium, they were treated either with blank NPs or with NPs containing γtcPNA4/donor DNA plus SCF. Two days later, we performed deep sequencing, revealing β-globin gene editing at position IVS2-654 at a frequency of 5.0% (Fig. 5b,c and Table 3). Six off-target sites with partial homology to the binding site of γtcPNA4 in the β-globin gene were also analysed, and extremely low off-target frequencies were found in two of the sites (0.000017 and 0.0000055%), with four sites showing no detectable sequence changes out of millions of reads (Table 3). Combined, we saw off-target mutations at an overall frequency of only 0.000012%, more than 400,000-fold lower than the 5% frequency of editing in human CD34+ cells.

**Discussion**

In the work reported here, we developed a gene editing strategy to correct a thalassaemia-associated mutation in the human β-globin gene and tested it in an established rodent model of human β-thalassaemia. Our results demonstrate that chemically modified, triplex-forming γPNAs and donor DNAs delivered intravenously via polymer NPs, and given in combination with SCF treatment, can mediate gene editing in vivo at a level sufficient to ameliorate the disease phenotype in the mice. We observed sustained reversal of the anaemia for up to 140 days post treatment, with normalization of serum haemoglobin concentrations and suppression of the reticulocytosis. We also saw a morphologic improvement in RBC cytology, along with reduced extramedullary haematopoiesis and reduction in splenomegaly. These findings suggest that our approach has the potential to yield a significant clinical response that could relieve the morbidity and mortality associated with β-thalassaemia.

There are two key advances for gene editing in this work. One is the incorporation of next generation PNA chemistry by substitution at the gamma position to enhance the binding properties of the PNAs. The second is the finding that the SCF/c-Kit pathway promotes increased gene editing by triplex-forming PNAs and donor DNAs. Upon ex vivo treatment of BM cells with γPNAs, the gene editing frequency in c-Kit+ cells was as high as 8%, 3-4-fold higher than with unmodified PNAs. The combination of SCF treatment with the γPNAs yielded even higher frequencies in the c-Kit+ cells, with just over 15% in a single treatment. In vivo, treatment of transgenic mice carrying a β-globin/GFP reporter transgene by i.p. injection of SCF followed by intravenous administration of PNAs containing γPNAs and donor DNAs yielded gene editing in CD117+ cells at frequencies up to 1% in a single treatment. In the thalassemic mouse model, simple intravenous injection of γPNAs/donor DNA NPs, plus SCF given i.p., resulted in a gene editing frequency of almost 4% in total BM cells (and 6.9% in Lin-Sca1+ cKit+ CD150+ CD135- cells, representing a sub-population of putative HSCs) and produced sustained amelioration of the disease phenotype, achieved in a minimally invasive manner without the need for stem cell harvest or transplantation.

Similarly, we achieved a frequency of 5% gene editing at the endogenous β-globin gene in human CD34+ haematopoietic progenitor cells treated once with SCF and γPNAs/donor DNA NPs ex vivo, and we showed that these cells can engraft into NOD-IL2rgnull mice with persistent evidence of gene editing, supporting the eventual translatability of our approach to individuals with thalassaemia.

Importantly, in a series of assays for haematopoietic colony formation, for induction of inflammatory cytokines, for generation of strand breaks and for off-target mutagenesis, there was essentially no measurable cellular toxicity and very low off-target effects from the γPNAs-containing NPs in either the mouse or human cells. In the human CD34+ cells, the frequency of off-target mutations at the 6 most closely homologous sites was 0.000012%, a value more than 400,000-fold lower than the gene editing frequency of 5%, providing a possible safety advantage relative to other gene editing approaches.

Two recent publications used either TALENs or CRISPR/Cas9 for gene editing in cell culture of human iPSC cells carrying the same IVS2-654 thalassaemia-associated β-globin mutation site as studied here. These publications achieved higher editing frequencies of 33% by TALENS and 12.3% by CRISPR/Cas9 in the other compared with our editing frequency in human CD34+ cells.

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**Table 1 | Off-target effects in bone marrow cells following intravenous treatment of β-globin/GFP mice with γtcPNA4/donor DNA NPs.**

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Sequences of partial homology (5’-3’)</th>
<th>Size of region sequenced</th>
<th>Alleles sequenced</th>
<th>Number modified</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-globin</td>
<td>TGGCCCTGAAAGAAAAGAGA</td>
<td>128</td>
<td>1,399,786</td>
<td>78,833</td>
<td>0.56</td>
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<tr>
<td>Vascular cell adhesion protein precursor 1</td>
<td>AGCCTGAAAGAAAAGAGA</td>
<td>111</td>
<td>480,013</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polypyrinidm tract binding protein</td>
<td>GACCTGAAAGAAAAGAGA</td>
<td>101</td>
<td>349,723</td>
<td>26</td>
<td>0.0074</td>
</tr>
<tr>
<td>Protocadherin fat 4 precursor</td>
<td>CACCCCTGAAAGAAAAGAGA</td>
<td>115</td>
<td>73,245</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Olfactory receptor 266</td>
<td>AAGCCCTGAAAGAAAAGAGA</td>
<td>172</td>
<td>1,092,990</td>
<td>2</td>
<td>0.00018</td>
</tr>
<tr>
<td>Syntaxin binding protein</td>
<td>AGAATGAAAGAAAAGAGA</td>
<td>150</td>
<td>2,478,636</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Muscleblind like protein</td>
<td>GGTTGGAAGAAAAGAGA</td>
<td>165</td>
<td>2,331,971</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ceruloplasmin isoform</td>
<td>AGACTGAAAGAAAAGAGA</td>
<td>154</td>
<td>1,390,439</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total off-target</td>
<td></td>
<td></td>
<td>8,197,017</td>
<td>28</td>
<td>0.00034</td>
</tr>
</tbody>
</table>

The top seven gene loci in the mouse genome with partial homology to the 18 bp γtcPNA4 target site in β-globin intron 2 were identified, with the sequences as indicated. β-globin/GFP mice were treated with SCF followed by intravenous injection with NPs containing γtcPNA4/donor DNA, and genomic DNA from c-Kit+ BM cells was subject to deep sequencing analyses at these loci. The size of the region sequenced around each site is listed, along with the number of alleles sequenced and the number of alleles with modified sequences.
Supplementary Figs 17 and 18. /C2 stain (H&E), wild-type mice and from thalassemic mice obtained 36 days after treatment with blank NPs or SCF plus α-globin gene editing. Horizontal bars within the boxes indicate mean; statistical analysis by Student’s unpaired t-test, *P < 0.05. (c) Reticulocyte counts (% of total RBCs) calculated in blood smears from thalassemic mice treated with blank NPs, SCF alone, SCF plus scrambled tcPNA4-Scr/donor DNA NPs, or with SCF plus tcPNA4/donor DNA NPs performed at the indicated number of days after treatment, up to 140 days. Data are presented as box and whisker plots showing the median and quartile range within each group over time (n = 6 per group). Only the SCF plus tcPNA4/donor DNA-treated mice achieved and maintained haemoglobin levels within the normal range during the duration of the experiment, reflecting the increased haemoglobin stability conferred by the gene editing. Horizontal bars within the boxes indicate mean; statistical analysis by Student’s unpaired t-test, *P < 0.05. (c) Reticulocyte counts (% of total RBCs) calculated in blood smears from thalassemic mice treated with either blank NPs or with NPs containing tcPNA4/donor DNA plus SCF on days 0 and 36 post treatment. Data are mean ± s.e.m., n = 3; statistical analysis by Student’s t-test, *P < 0.05. (d) Images of spleens from wild-type mice or thalassemic mice treated with blank NPs, SCF alone, SCF plus scrambled tcPNA4-Scr/donor DNA NPs or SCF plus tcPNA4/donor DNA NPs at 36 days after the last treatment. Average spleen weights (± standard errors, n = 3; except for wild-type, where n = 1) are listed below the images. Splenomegaly is corrected only by SCF plus tcPNA4/donor DNA treatment. Scale bar, 1.0 cm. (e) Histopathologic analysis of spleen sections from wild-type mice and from thalassemic mice obtained 36 days after treatment with blank NPs or SCF plus tcPNA4/donor DNA NPs. Haematoxylin and eosin stain (H&E), × 40 magnification. Scale bar, 10.0 μm. Additional images of spleens from other treatment groups and with additional stains are presented in Supplementary Figs 17 and 18.

of 5% (albeit a different cell type). With respect to off-target effects, these publications used a PCR-based T7E1 assay, and so quantitative comparisons with our deep sequencing results are not possible. Also, these publications did not test their approaches in vivo, and so we cannot make comparisons to our in vivo results in the thalassemic mice.

CD117 is the product of the c-Kit gene and is a receptor tyrosine kinase that mediates downstream signalling. Our results suggest that activation of this pathway promotes gene editing. Mechanistically, we observed elevated DNA repair gene expression in CD117 cells, including factors that may play a role in triplex-induced gene editing4,10,26,27. Importantly, we show
Eight weeks after transplantation, BM cells were harvested from the mice and human CD34+ cells that were harvested from NOD-scid IL2rnull mice were transplanted into NOD-scid IL2rnull mice. This effect has been attributed to cells in the marrow having produced much higher proportions of chimerism at one ratio of wild-type donor to thalassemic recipient cells. Overall, our results provide motivation for further development of NP-mediated delivery of γPNAs and donor DNAs as a possible therapeutic strategy to achieve in vivo gene editing for treatment of human genetic disorders. The work here demonstrates gene editing in BM, but other work from our group has shown that NP delivery of PNAs to lung airway epithelia is also possible to achieve correction of the CFTR gene mutation associated with cystic fibrosis. In this regard, the effect of SCF on gene editing in BM raises the possibility that other cytokines that CD117+ cells show functional increases in DNA repair and that treatment with SCF produces further increases.

The 4% frequency of gene editing in total BM cells (and 6.9% in Lin-Scal+cKit+CD150+CD135- putative HSCs) achieved in the thalassemic mice was sufficient to achieve a clear improvement in phenotype. That gene correction at these frequencies could confer a phenotypic impact is consistent with transplantation studies in thalassemic mice and in patients in which mixed chimerism at one ratio of wild-type donor to thalassemic recipient cells in the marrow has produced much higher proportions of donor RBCs in the periphery. This effect has been attributed to increased survival of genetically corrected erythroblasts during erythropoiesis, decreased ineffective erythropoiesis and increased survival in the circulation of corrected erythrocytes relative to thalassemic RBCs.

Overall, our results provide motivation for further development of NP-mediated delivery of γPNAs and donor DNAs as a possible therapeutic strategy to achieve in vivo gene editing for treatment of human genetic disorders. The work here demonstrates gene editing in BM, but other work from our group has shown that NP delivery of PNAs to lung airway epithelia is also possible to achieve correction of the CFTR gene mutation associated with cystic fibrosis. In this regard, the effect of SCF on gene editing in BM raises the possibility that other cytokines...
or growth factors could similarly serve to boost gene editing potential not only in BM but also in other tissues. In addition, the ability of SCF (which is an established activator of HSCs and is in clinical use) to stimulate gene editing by triplex-forming PNAs may also be applicable to other editing methods, such as CRISPR/Cas9.

Further improvements in PNA-mediated gene editing are still possible via additional PNA chemical modifications. Most encouraging at this point, however, is that even though 5′PNAs show improved gene editing potency, the off-target effects in the genome remain extremely low. This is in accordance with the lack of intrinsic nuclease activity in PNAs, and reflects the mechanism of triplex-induced gene editing, which creates an altered helix that engages endogenous DNA repair pathways.

The sequences of PNAs used in this study are given in Fig. 1. The single-stranded donor DNA oligomer was prepared by standard DNA synthesis except for the inclusion of three phosphorothiate internucleoside linkages at each end to further facilitate detection by deep sequencing. The sequences of the donor DNA matches positions 624–684 in β-globin intron 2 and is as follows, with the correcting IVS2-654 mutation, and so the donor was designed to introduce rather than correct the IVS2-654 mutation, and so the sequence of the donor was:

\[
\text{AAAGATAACAGTGATAATTTCTGGGTT}
\]

Table 2 | Off-target effects in bone marrow cells following intravenous treatment of β-thalassemic mice with SCF and γtcPNA4/donor DNA NPs.

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Sequences of partial homology (5′-3′)</th>
<th>Size of region sequenced</th>
<th>Alleles sequenced</th>
<th>Number modified</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-globin</td>
<td>TGCCTGAGAAGAAAGAGA</td>
<td>128</td>
<td>8,615,313</td>
<td>337,192</td>
<td>3.9</td>
</tr>
<tr>
<td>Vascular cell adhesion protein precursor 1</td>
<td>AGCCTGAGAAGAAAGAGA</td>
<td>111</td>
<td>482,051</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Polyadenosine tract binding protein</td>
<td>GAACCTGAGAAGAAAGAGA</td>
<td>101</td>
<td>355,567</td>
<td>2</td>
<td>0.00056</td>
</tr>
<tr>
<td>Protacodera fat 4 precursor</td>
<td>CACCCCTGAGAAGAAAGAG</td>
<td>115</td>
<td>123,158</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Olfactory receptor 266</td>
<td>AAGCCTGAGAAGAAAGAGGA</td>
<td>172</td>
<td>1,099,880</td>
<td>262</td>
<td>0.0231</td>
</tr>
<tr>
<td>Syntaxin binding protein</td>
<td>AGAAATGAAAGAAAGAGA</td>
<td>150</td>
<td>2,493,024</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Musclebind like protein</td>
<td>GAGGTGAGAAGAAAGAGA</td>
<td>165</td>
<td>2,336,715</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ceruloplasmin isoform</td>
<td>AGGACTGAGAAGAAAGAG</td>
<td>154</td>
<td>1,397,271</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total off-target</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number sequenced</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alleles sequenced</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number modified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The top seven gene loci in the mouse genome with partial homology to the 18 bp γtcPNA4 target site in β-globin intron 2 were identified, with the sequences as indicated. Thalassemic mice were treated with SCF followed by intravenous infusion with NPs containing γtcPNA4/donor DNA, and genomic DNA from c-Kit + BM cells was subject to deep sequencing analysis at these loci. The size of the region sequenced around each site is listed, along with the number of alleles sequenced and the number of alleles with modified sequences.

Table 3 | Deep sequencing analysis of targeted gene editing versus off-target effects in human CD34+ hematopoietic cells following ex vivo treatment with SCF and γtcPNA4/donor DNA NPs.

<table>
<thead>
<tr>
<th>Gene locus</th>
<th>Sequences of partial homology (5′-3′)</th>
<th>Size of region sequenced</th>
<th>Alleles sequenced</th>
<th>Number modified</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-globin</td>
<td>TGCCCTGAGAAGAAAGAGA</td>
<td>128</td>
<td>12,489,910</td>
<td>606,230</td>
<td>5.02</td>
</tr>
<tr>
<td>Serine Threonine Kinase</td>
<td>ATTCTGAGAAGAAAGAC</td>
<td>189</td>
<td>3,330,879</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Anoctamin-3</td>
<td>AATCTGAGAAGAAAGAC</td>
<td>150</td>
<td>5,805,518</td>
<td>1</td>
<td>0.000017</td>
</tr>
<tr>
<td>39s Ribosomal protein L17</td>
<td>AGCCCTGAGAAGAAAGA</td>
<td>169</td>
<td>4,211,251</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Neuroblast differentiation</td>
<td>TCCCTGAGAAGAAAGA</td>
<td>198</td>
<td>3,579,389</td>
<td>2</td>
<td>0.000555</td>
</tr>
<tr>
<td>Transcription enhance factor</td>
<td>TCTCTCGAGAAGAAAGA</td>
<td>244</td>
<td>80,548</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rho GTPase activating protein</td>
<td>CAACCTGAGAAGAAAGA</td>
<td>154</td>
<td>8,256,220</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total off-target</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number sequenced</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alleles sequenced</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number modified</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The top six gene loci in the human genome with partial homology to the 18 bp γtcPNA4 target site in β-globin intron 2 were identified, with the sequences as indicated. Human CD34+ hematopoietic cells were treated ex vivo with SCF and with NPs containing γtcPNA4/donor DNA, and 2 days later genomic DNA from the cells was subject to deep sequencing analysis at these loci. The size of the region sequenced around each site is listed, along with the number of alleles sequenced and the number of alleles with modified sequences. The sequences of PNAs used in this study are given in Fig. 1. The single-stranded donor DNA oligomer was prepared by standard DNA synthesis except for the inclusion of three phosphorothiate internucleoside linkages at each end to protect from nucleosome degradation. The sequence of the donor DNA matches positions 624–684 in β-globin intron 2 and is as follows, with the correcting IVS2-654 nucleotide underlined:

\[
\text{AAAGATACACGTGATAAATTCTGGGTT}
\]

For gene editing experiments in the human CD34+ studies, the donor DNA was designed to introduce rather than correct the IVS2-654 mutation, and so the sequence of the donor was:

\[
\text{AAAGATAACAGTGATAATTTCTGGGTT}
\]

Note that besides the IVS2-654 mutation, this donor region sequenced around each site is listed, along with the number of alleles sequenced and the number of alleles with modified sequences.

**Methods**

**Oligonucleotides.** All MP-PNA monomers were prepared from Boc-(2-(2-methoxycarbonyl)-ethyl)-serine as a starting material by a series of multistep synthetic procedures including reduction, mitsunobu reaction, nucleoside (A,C,G and T) conjugation and then ester cleavage. At each step the respective product was purified by column chromatography. PNA oligomers were synthesized on solid support conjugation and then ester cleavage. At each step the respective product was purified by column chromatography (acetonitrile:water) and characterized with a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer.

**PLGA nanoparticle synthesis and characterization.** PLGA nanoparticles containing PNAs and DNAs were formulated using a double-emulsion solvent evaporation method and characterized PLGA nanoparticles encapsulating PNA/donor DNA were formulated using a double-emulsion solvent evaporation technique. PNAs and donor DNAs were dissolved in 80:2 μl DNA:water-free water. All nanoparticle batches had 2 nmole mg−1 of PNA or γPNA and 1 nmole mg−1 of donor DNA. The encapsulant was added dropwise to a polymer solution containing 80 mg/50:50 ester-terminated PLGA dissolved in dichloromethane (800 μl), then ultrasonicated (3 × 10 s) to form the first emulsion. To form the second emulsion, the first emulsion was added slowly dropwise to 1.6 ml of 5% aqueous polyvinyl alcohol and then ultrasonicated (3 × 10 s). This mixture was finally poured into 20 ml of 0.3% aqueous polyvinyl alcohol and stirred for 3 h at room temperature. Nanoparticles were then thoroughly washed with 20 ml water (3 × 3) and further collected each time by centrifugation (12,000 rpm for 10 min at 4 °C). Nanoparticles were resuspended in water, frozen at −80 °C, and then lyophilized. Nanoparticles were stored at −20 °C after lyophilization. Nucleic acid release was analysed by incubating nanoparticles (4–6 mg) in...
phosphate-buffered saline (PBS; 600 µl) in a 37°C shaker, spinning down and removing supernatant. Further absorbance of the supernatant was measured at 260 nm at the indicated time points. At 24 and 48 h nanoparticles, the residual nucleic acid in the nanoparticle pellet was extracted and total nucleic acid content was calculated as a sum of absorbance obtained from the pellet as well as supernatant. Absorbancess at 260 nm were measured with a Nanodrop 2,000 (ref. 21).

Ex vivo experiments with BM cells from β-globin/GFP mice. BM cells were harvested by flushing femurs and tibias of β-globin/GFP transgenic mice with Rosewell Park Memorial Institute (RPMI)/10% foetal bovine serum (FBS) media. Nanoparticles (2 mg mL⁻¹) were used to treat ~300,000–500,000 cells for 48 h in RPMI/10% FBS media containing glutamine, in triplicate samples. After 48 h, cells were fixed with 4% paraformaldehyde, and analysed by flow cytometry. Cells treated with blank nanoparticles and tibias were included as controls.

For experiments with CD117⁺ cells, were isolated by magnetic separation and grown in cells. Iscove’s Modified Dulbecco’s Media containing insulin (10 ng mL⁻¹), FCS (10%) and erythropoietin (1 U mL⁻¹). Where indicated, 3 µg mL⁻¹ of SCF (recombinant murine SCF, catalogue #250-03, PeproTech, Rocky Hill, NJ) was added before nanoparticle treatment. NPs (2 mg mL⁻¹) were used to treat 50,000–100,000 CD117⁺ cells in triplicate for 48 h in the above media, followed by flow cytometry analyses as above. Inhibitors were used at concentrations of 200 nM (dasatinib), 1.0 µM (MEK162) and 3.0 µM (BKM120). Dasatinib was obtained from Cayman Chemical (Ann Arbor, MI; item #11498) and dissolved according to manufacturer’s protocol. MEK12 and BKM120 were obtained from Dr. Harriet Kluger, Yale University.

Sorting and flow cytometry of cells from β-globin/GFP mice. BD Bioscience kit catalogue #558451 (BD IgM Haematopoietic Progenitor Stem Cell Enrichment Set—DM) was used to isolate CD117 cells. Enrichment for CD117 was confirmed by flow cytometry. CD117⁺ enriched cells were labelled with CD117-APC (BD Pharmingen catalogue #558451) antibody used a dilution of 1:100. Cells were co-labelled with an antibody (anti-integrin β3, BD Pharmingen catalogue #555746) used a dilution of 1:100 for gating purposes. To quantify GFP expression, after CD117 co-labelling, flow cytometry was performed using FACScalibur by resuspending cells in PBS/1%FBS where green fluorescent cells are measured in the FL1 channel and APC stained cells are in the FL4 channel. Antibodies for other markers were Ter119 (BD Pharmingen catalogue #561018) and CD45 APC (BD Pharmingen catalogue #561018), both used a dilution of 1:100. See below for isolation of progenitor cell populations from thalassemic mice.

DNA binding gel shift assays. For gel electrophoresis, synthetic 120 bp dsDNA targets were incubated with indicated oligomers at 37°C in low ionic strength buffer (10 mM NaPi, pH 7.4). The samples were separated on 10% non-denaturing polyacrylamide gels in 1 × TBE buffer. The gels were run at 100 V cm⁻¹ for 1 h. After electrophoresis, the gels were stained with 1 × SYBR Gold (catalogue #S11494, Invitrogen) for 10 min, washed 2 × with 1 × TBE buffer, and then imaged using a gel documentation system (BioDoc-It System). The images were then inverted using Adobe Photoshop 6.0.

Comet assay. 400,000 BM cells per well were plated on 6-well plates in 1 mL media, then treated with 2 mg mL⁻¹ of either blank NPs or PLGA NPs containing either α-tropinA/donor DNA or ftb-muscleblind-like RNA as an internal control, as indicated in Supplementary Fig. 4. After 48 h, cells were harvested, and prepared using the Trevigen Comet Assay kit per manufacturer’s protocol (Trevigen, Gaithersburg, MD). Briefly, cells were suspended in agarose, added to comet slides, and dried. Samples were then inverted using an DEER microscope. TriTek Comet Score freeware was used to analyse images.

Reporter gene assay for homology-dependent repair. An inactivating I-Sce1 site was cloned 36 amino acids into the firefly luciferase open reading frame under the control of a cytomegalovirus (CMV) promoter. The reporter construct also contains a promoterless luciferase gene used as a template for homologous recombination. A DSb in the luciferase reporter is created by in vitro digestion with the I-Sce1 restriction enzyme (NEB #R0694L). Plasmid DNA was digested with I-Sce1 for 1 h at 37°C at a ratio of 10 units enzyme to 1 µg DNA and then the enzyme was inactivated at 65°C for 20 min. The linearization of the plasmid was confirmed for each digestion via gel electrophoresis and the linear plasmid was purified using the Qiagen Qiagen spin columns. After separation, CD117⁺ and CD117⁻ cells from BM of β-globin/GFP transgenic mice, cells transfected using the Lonza 2b Nucleofector Device. 5 × 10⁶ cells were transfected with 1 µg of either the luciferase reporter vector or a positive control firefly luciferase expression vector, to test for a resultual luciferase gene plasmid transfection efficiency control. All transfections were performed in triplicate. After transfection the cells were plated at a density of 5 × 10⁴ cells per ml in 12-well plates. After 24 h incubation post transfection, luciferase activity was measured using the Promega Dual Luciferase Assay Kit. In each sample firefly luciferase activity was normalized to the renilla luciferase transfection control. Reporter reactivation was calculated as a ratio of normalized firefly luciferase activity in the cells transfected with the reporter plasmid to the positive control.

Mouse models and in vivo treatments. All animal use was in accordance with the guidelines of the Animal Care and Use Committee of Yale University and confirmed to the recommendations in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council. National Academy of Sciences, 1996).

The β-globin/GFP transgenic mice were obtained from Ryszard Kole, University of North Carolina10. For treatment of the mice, where indicated SCF (220 µg kg⁻¹ per mouse, Recombinant Mouse SCF, carrier-free, R&D catalogue #455-mc-050/CF) was injected intraperitoneally 3 h before treatment with 4 mg of NPs in 150 µl PBS delivered via retro-orbital intravenous injection. Each mouse received four treatments given at 48 h intervals. Mice were anaesthetised with isoflurane followed by retro-orbital bleeding (~100 µl) using ethylendiaminetetraacetic acid–treated glass capillary tubes. The blood was evacuated into tubes with 5 µl of 0.5 M EDTA acid in heparinized coated tubes. Complete blood counts were performed using a Hemavet 950ES (Drew Scientific, Oxford, CT) according to the manufacturer’s protocol. Slides containing blood smears were stained with Wright and Giemsa stain for evaluation. Methylene blue staining was used for RNA. RNA samples were sequenced by the Illumina HiSeq with 75 paired-end reads at the University of North Carolina19. For treatment of the mice, where indicated SCF (recombinant murine SCF, catalogue #250-03, PeproTech, Rocky Hill, NJ) was injected intraperitoneally 3 h before treatment with 4 mg of NPs in 150 µl PBS delivered via retro-orbital intravenous injection. Each mouse received four treatments given at 48 h intervals. Mice were anaesthetised with isoflurane followed by retro-orbital bleeding (~100 µl) using ethylendiaminetetraacetic acid–treated glass capillary tubes. The blood was evacuated into tubes with 5 µl of 0.5 M EDTA acid in heparinized coated tubes. Complete blood counts were performed using a Hemavet 950ES (Drew Scientific, Oxford, CT) according to the manufacturer’s protocol. Slides containing blood smears were stained with Wright and Giemsa stain for evaluation. Methylene blue staining was used for RNA. RNA samples were sequenced by the Illumina HiSeq with 75 paired-end reads at the Yale Center for Genome Analysis. Samples were analysed as previously described12. Primers for deep sequencing were designed using Primer3 database. The primers used for β-globin intron 2 were as follows: forward primer: 5'-GATTTATGTGCACTATGCACT-3' and 5'-AATTGGAAGGGTCAGTCATGA-3'; poly-I-UTR tract binding protein (5'-CCACATCGATTGATATGAGC-3' and 5'-TATCTAAGGCATGCTTCTAG-3'); palindromic-like protein (5'-TCATCAACTTGTCAGTCCTC-3' and GACATCATATCGTCTCAAG-3'); oligo octamer 266 (5'-CCCTGATAGGGTCTGATGGAAGG-3' and 5'-GGTGATGCTAACTG-3'); syntaxin isoform 5 (5'-GACCTGCTGCTCATC-3' and 5'-TCTGATGCTTTCCAC-3'); muskel-like protein (5'-GCATTGGTCTGAGATCCT-3' and 5'-GATCGACAATAGGACCACC-3'); cefuroximase isoform (5'-ATGCGAGAAGAAC-3' and 5'-TCTGAGGTTCCTACCGAG-3').

Cytokine array analysis. NPs (4 mg) containing γ-tCRNA/donor DNA were injected iv. into β-globin/GFP transgenic mice. After, and after 48 h, plasma
samples were collected. Cytokine array analyses were performed on 25.0 μl of plasma and analysed for cytokines using the luminex based cytokine detection technique at the Cytoplex Core Facility at Yale University (https://medicine.yale.edu/obgyn/dss/immunology/).

**Analysis of HSCs from BM of treated thalassemic mice.** Thalassemic mice were treated as above with four treatments of NPs at 2 day intervals of 4 mg of NPs in 150 μl PBS delivered via retro-orbital intravenous injection along with SCF given i.p. Sixty-five days later, mouse tailbius were collected and crushed in mortar with 5 μl of 3-fluorescein activated cell sorting (FACS) buffer (PBS + 5% fetal serum albumin + 2 mM EDTA). The BM was passed through a 100 μm cell strainer and then the cells were collected by centrifugation at 250g for 5 min at room temperature. The cell pellets were resuspended in 5 ml of RBC lysis buffer for 1 min. The suspensions were diluted with 1 ml PBS and 14 ml FACS buffer, cells were collected by centrifugation, and then resuspended in FACS buffer for antibody staining, followed by FACS. Antibodies used were as follows: BD Biosciences (www.bdbiosciences.com): BD533086 (CD45R2B2D0), BD533309 (CD11b), BD533562 (Ter119), BD533506 (CD3ε), BD533502 (CD8α), BD533549 (CD4); BD567297-6A/7-5EB (Stemcell Technologies, Vancouver, British Columbia, Canada). Cells were harvested, pelleted and resuspended in Stemspan serum-free expansion medium (SFEM) with StemSpan CC100 cytokine mixture (Stemcell Technologies, Vancouver, British Columbia, Canada). Cells were treated with NPs at 4 mg of NPs in 200 μl of SFEM with stemSpan CC100 for 24 h, followed by 100% methanol for 30 min at room temperature. The cell pellets were resuspended in 50% acetone/50% methanol for 15 min at room temperature, followed by 1% paraformaldehyde/2% sucrose for 15 min at room temperature, followed by 100% methanol for 30 min at −20°C and 50% methanol/50% acetone for 20 min at 10°C. Slides were then incubated in permeabilization/blocking solution (10% BGS, 0.5% Triton X-100 in PBS) at room temperature for 1 h. Primary antibody (phospho-specific H2A.X (Cell Signaling #9718S)) was diluted 1:500 in permeabilization/blocking solution and stained for 1 h. The secondary antibody was Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (IgG; Life Technologies). Cells were stained with DAPI to visualize the nuclei.

**Transplantation into adult BM.** BM was obtained from recipient NOD.Cg-PrkdcscidIL2rgm1Wjl-scid null (scid) mice. BM was collected from the research colony maintained at the Jackson Laboratory. The suspensions were diluted with 1 ml FBS and 14 ml FACS buffer, cells were centrifuged and the cells were collected by centrifugation at 250g for 5 min at room temperature. The cell pellets were resuspended in 5 ml of RBC lysis buffer for 1 min. The suspensions were diluted with 1 ml PBS and 14 ml FACS buffer, cells were collected by centrifugation, and then resuspended in FACS buffer for antibody staining, followed by FACS. Antibodies used were as follows: BD Biosciences (www.bdbiosciences.com): BD533086 (CD45R2B2D0), BD533309 (CD11b), BD533562 (Ter119), BD533506 (CD3ε), BD533502 (CD8α), BD533549 (CD4); BD567297-6A/7-5EB (Stemcell Technologies, Vancouver, British Columbia, Canada). Cells were treated with NPs at 4 mg of NPs in 200 μl of SFEM with stemSpan CC100 for 24 h, followed by 100% methanol for 30 min at room temperature. The cell pellets were resuspended in 50% acetone/50% methanol for 15 min at room temperature, followed by 1% paraformaldehyde/2% sucrose for 15 min at room temperature, followed by 100% methanol for 30 min at −20°C and 50% methanol/50% acetone for 20 min at 10°C. Slides were then incubated in permeabilization/blocking solution (10% BGS, 0.5% Triton X-100 in PBS) at room temperature for 1 h. Primary antibody (phospho-specific H2A.X (Cell Signaling #9718S)) was diluted 1:500 in permeabilization/blocking solution and stained for 1 h. The secondary antibody was Alexa Fluor 488-conjugated goat anti-rabbit immunoglobulin G (IgG; Life Technologies). Cells were stained with DAPI to visualize the nuclei.

**Cytokine microarray analysis, after NP treatment of the CD34+ cells, as above, supernatant medium was collected and analysed by a luminex based assay as describe above.**

**Western blot analysis.** Western blot analysis was performed as above with a modification of genomic β-globin sequences in human hematopoietic stem/progenitor cells. 213–224 (2006).

**Transplantation of treated human CD34+ cells.** Human CD34+ cells (6 × 10^6) were treated, as above, with SCF (1.0 μg) and 2.0 mg of nanoparticles containing γcPNA4/donor DNA in the SFEM with the CC100 cytokine mixture for 48 h and then were used to transplant immunodeficient recipient NOD-scid IL2rnull mice. As above, all animal use was in accordance with the guidelines of the Institutional Animal Care and Use Committees (IACUC) of the Massachusetts Medical School, Yale University, and The Jackson Laboratory. NOD.Cg-PrkdcscidIL2rgm1Wjl-scid (abbreviated NOD-scid IL2rnull) mice were obtained from the research colony maintained at The Jackson Laboratory. Transplant of treated CD34 cells into the NOD-scid IL2rnull mice was performed by intracardiac injection into newborn mice as previously described. Engraftment was confirmed at 7 weeks. Eight weeks post transplant, human CD34+ cells were isolated from mouse BM using EasySep human CD34+ positive selection Kit (StemCell Technologies; Cat# 18068). Genomic DNA was extracted from CD34+ cells using the Wizard Genomic Purification Kit (Promega). Samples were submitted for deep sequencing as described above.

**Data availability.** Microarray data have been deposited in Gene Expression Omnibus ( GEO) database (http://www.ncbi.nlm.nih.gov/geo/) under accession code GSE86859.

References:


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Author contributions


Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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