nature portfolio

Peer Review File

Precise genomic integration of large DNA fragments by donor-directed annealing using prime editing

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This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Biotechnology.

Parts of this Peer Review File have been redacted as indicated as we could not obtain permission to publish the reports from one of the peer reviewers.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In the revision, the authors have supplemented important data to support the efficiency of PA in primary human T cells and in mouse liver, which has substantially improved the manuscript. I suggest acceptance of this revised manuscript.

(Remarks on code availability)

Author Rebuttal letter:

Dear anonymous reviewers,

Previously, we had submitted this manuscript to Nature (2025-09-24072A), but it was ultimately rejected after two rounds of revisions. Now, it has been transferred to Nature Biotechnology. We hope it will be re-evaluated according to the evaluation criteria of Nature Biotechnology.

Please find our detailed responses to the reviews shown in blue below. Revised sections of the main text are colored red in the revised manuscript.

We would like to inform the three reviewers of the following changes performed during this revision process in common.

1) The title was changed from “Site-specific replacement of large-scale DNA fragments in human cells” to “Programmable replacement of large-scale DNA fragments in human cells”, to emphasize the programmability of prime assembly technology.
2) We have improved the prime assembly efficiency in human primary T cells from the previous 2% level to a maximum of 28% by optimizing our protocol, such as buffer conditions.
3) We newly conducted prime assembly experiment on mice in vivo, and the results have been added to Revised Figure 5f and Supplementary Figure 9. So, four co-authors (Hyeon Woo Im, Doyoon Kim, Soyoon Lee, and Yohan Kim) who performed these in vivo mouse experiments have been involved during the revision process.

Thank you very much.

Sincerely yours,
Sangsu Bae, Ph. D.

Reviewer's Comments:

Referee #1 (Remarks to the Author):

The authors have performed more experiments and analyses, and the study has been substantially improved. I suggest the acceptance of this revised manuscript.

Response: We sincerely thank the reviewer for the positive evaluation of our revised manuscript. We are grateful that the additional experiments and analyses were considered to have substantially improved the study. Furthermore, we thank the reviewer for the supportive and fair assessment in this highly competitive gene-editing field.

Referee #2 (Remarks to the Author):

The revised manuscript has supplemented several important data to improve this study. However, there are several major concerns.

Response: We appreciate the reviewer's careful evaluation and constructive comments. We have done our best to address each concern point by point, and revise the manuscript accordingly, as below.

Comments:

1. The core strategy of using prime editing to generate a 3' flap in the donor DNA was reported in PAINT two years ago. The donor template of SF-PA and SFn-PA is very similar to PAINT3, which compromises the novelty of this study. TWIN-PE sets up the idea to generate complementary flaps to delete large DNA fragment and insertion of short DNA fragments. PAINT sets up the idea of generate flaps in donor DNA. PA seems pretty much like the combination of twinPE and PAINT. It suggests that the conceptual novelty is limited.

Response: Thank you for the important comment. We agree that twinPE and PAINT demonstrated key concepts related to PA. However, while PAINT systems still rely on target DNA cleavage, PA is a tool independent of DNA double-strand breaks (DSBs). Conversely, twinPE uses a pair of pegRNAs, but because it does not use donor DNAs, large-scale DNA insertions or replacements are not possible. Of course, some might say that prime assembly might be an extension of existing technologies, but this is the first case to achieve such results, large-scale DNA replacements, in human cells with high efficiency (~70%), with highly rare off-target events.

Please note that two independent groups have recently reported conceptually similar studies in Nature [a quadruple-pegRNA approach by Shi et al. and Prime Assembly by Liu et al.], implying that this PA system is being evaluated as a new and innovative technology. Please also note that we first submitted this study to the journal Nature last September (2025). Therefore, we would like to emphasize that this study is contemporary, not a follow-up to the recently published studies.

2. Although the authors compared PAs with PAINT in a AAVS1, the efficiency of PAINT in this study is much lower than in the previously reported study. Although the efficiency is site-dependent and each lab has their own expertise, the insertion efficiency of PA is not superior than PAINT (compared the data of PA in this study and the PAINT data in Cell Discov., 2023). For example, PAINT reached up to 85% efficiency for a 2.5-kb donor. More importantly, in a application scenario, PAINT demonstrated ~60% insertion of a CD19 CAR-EGFP transgene cassette in primary T cells, resulting in functional CAR-T cells with specific tumor-killing activity. But in the revision, CAR expression was observed in 1.4% of cells with F-PA, 2.5% with SFn-PA, and 2.3% with DF-PA. Such low efficiency has no application and transitional potential. Cas9-mediated HDR in primary T cells can reach 20-30% with naked dsDNA template and over 50% with ssDNA template delivered by AAV6.

Response: Thank you for the comments. In fact, our research team specializes in gene editing, but we have never worked with primary T cells. This is the reason we obtained low editing efficiencies (1~3%) in primary T cells during the first revision process.

However, during this revision process, we sought to optimize PA treatment in T cell experiments. In particular, we found that the low survival rate of DNA-introduced T cells was likely a major bottleneck. We therefore improved cell recovery by optimizing the electroporation condition using B1mix buffer [PMID: 37500747] and modifying the post-electroporation culture conditions. As a result, CAR integration efficiencies were significantly improved across all PA systems, reaching 8.3% with SF-PA, 19.1% with SFn-PA, and 28.1% with DF-PA. Importantly, we confirmed that, under these conditions, electroporated T cells showed viability, apoptosis levels, and proliferation rates similar to control T cells electroporated with PE7 mRNA alone, and increased about 20-fold within 7 days to proliferate to more than 10^7 cells. We have added these results to revised Figure 5a-5e and description in the revised manuscript [Lines 298–316].

Updated Figure 5a-5c.

3. In patient-derived fibroblasts, an average replacement efficiency of 1.9% for SF-PA, 2.4% for SFn-PA, and 1.3% for DF-PA was observed. It is really confusing why PA functions very well in HEK293T cells but not in primary human cells. Such low efficiency in primary human cells makes the prospects for translational application are highly unfavorable.

Response: Thank you for the valuable comment. We agree that the low PA-mediated replacement efficiency observed in patient-derived fibroblasts in the previous version was insufficient to support strong clinical applicability in that cell type. During this revision process, we found that the condition of patient-derived fibroblast lines was not good, compared to that of healthy individuals. Therefore, we decided to remove this data in the revised manuscript. Instead, we focused on improving primary human T-cell experiments and obtained significantly improved CAR integration efficiencies across all PA systems, reaching 8.3% with SF-PA, 19.1% with SFn-PA, and 28.1% with DF-PA, as described above #2.

On the other hand, to evaluate the in vivo applicability of PA, we prepared four plasmids containing PE7, two pairs of pegRNAs, and GFP donor, and all plasmids were subsequently delivered to mice via hydrodynamic tail-vein injection. Seven days after injection into mice, liver tissue was sorted by GFP fluorescence, and the average insertion efficiencies of 1.1% and 4.3% for DF-PA and SFn-PA, respectively. We have added these results to revised Figure 5f and Supplementary Figure 9 in the revised manuscript [Lines 317–327].

Newly added Supplementary Figure 9

Finally, to demonstrate the in vivo applicability of PA, we evaluated PA-mediated editing efficiency in mouse hepatocytes. Prior to in vivo experiments, two pegRNAs were integrated into a single plasmid to minimize the number of constructs required for delivery. We prepared four plasmids containing PE7, two pairs of pegRNA, and a GFP donor (Supplementary Fig. 9). Then, these plasmids were delivered to mice via hydrodynamic injection through the tail vein to induce PA editing in the liver. After harvesting the liver, hepatocytes were isolated, and GFP-positive hepatocytes which represented successful transfection, were enriched by fluorescence-activated cell sorting and then subjected to genomic analysis. Among the GFP-positive hepatocytes, the average target insertion efficiencies of SFn-PA and DF-PA were 4.3% and 1.1%, respectively (Fig. 5f). Sequencing analysis of individual reads further confirmed precise junction formation (Supplementary Fig. 9). These results demonstrate the feasibility of PA-mediated targeted DNA integration in vivo.

4. The underlying mechanism of PA is quite important for further optimization, but it is not well characterized. Although the authors used lots of inhibitors and siRNAs to target key components of DNA repair pathways, what the main pathway or pathways involved in PA-mediated replacement and how it occurs are not clear. As Rad51 is critical, does PA partially use the HDR pathway? In the manuscript there is no clear conclusion of how PA works. For the cell cycle arrest experiments, it seems that G1 arrest dramatically (reduced to less than 50% at the tested targets of all PAs) inhibited PA activity (Suppl. Fig. 6c), but why do the authors claim “Although PA efficiency was relatively reduced compared to untreated controls, integration was consistently observed under the G1-phase arrested condition.”? Since the inhibitor treatment only increased 20% (35% without treatment vs 55% with treatment), such dramatic reduction of PA activity suggests that PA mainly functions during S/G2 phase. If it is

true, PA may not suitable for non-dividing cells, which would have minimal efficiency for in vivo gene therapy. In this point, eePASSIGE might have advantages against PA for in vivo gene therapy.

Response: We thank the reviewer for this important comment. We agree that understanding the cellular mechanism of PA is important for further optimization. In this study, the inhibitor and siRNA experiments suggested that several DNA repair-associated factors, including RAD51, BRCA1, BRCA2, LIG3, and DNA2, could modulate PA activity, indicating that PA-mediated gene replacement is influenced by multiple repair processes, rather than a single pathway. This is supported by the fact that we have demonstrated high PA efficiency (~28%) in primary human T cells.

We also agree that cell cycle conditions can affect PA efficiency. In G1 arrest experiments, we confirmed that PA activity decreased under cell cycle arrest conditions, and thus we have revised the manuscript to clarify this point in the revised manuscript, as follows [Lines 244–246].

Although PA efficiency was relatively reduced compared to untreated controls, proliferating cells showed relatively high PA activity, suggesting that PA may partially depend on S/G2 phase-related repair processes.
On the other hand, we would like to point out that similar results have been found in two already published papers. Both the Wen Xue group and the Hao Yin group showed slightly reduced PA efficiency in G1 arrested cells compared to untreated cells, but they described that it is generally not significantly affected by the cell cycle.

Figure for the reviewer only.
Referee #3 (Remarks to the Author):

While Jung et al. have addressed several points raised in the previous review, key concerns regarding the comparative performance and practical utility of the Prime Assembly (PA) method remain insufficiently resolved.

Response: We thank the reviewer for the careful evaluation of our revised manuscript. To more clearly demonstrate the practical utility of the Prime Assembly (PA) method, we improved the PA efficiency in human primary T cells from the existing 2% level to up to 28% through protocol optimization. Additionally, we performed new PA experiments in vivo using mice and added the results to the revised manuscript.

Comments: The authors have compared the integration efficiency of PA with other state-of-the-art methods. The reviewer notices that HMEJ, a DSB-dependent approach, achieved comparable efficiency to PA. While this establishes parity in efficacy, the presumed advantages of PA likely lie in its precision and safety profile as a DSB-free strategy. To substantiate this claim, a comprehensive comparison of editing outcomes between PA and HMEJ (and, if feasible, PASSIGE) is essential. This should include detailed analyses of on-target precision, indel frequencies, structural variants, and any off-target integration events to objectively demonstrate whether PA offers a superior performance balance.

Response: Thank you for the comment.

To address this concern, we performed Oxford Nanopore long-read sequencing of HMEJ-treated samples to characterize on-target integration products. To analyze the HMEJ-mediated integration results in more detail in the kb range, Oxford nanopore sequencing was performed on the HMEJ-treated samples. Precise integration was detected in 7.7% of total reads, while impaired integration and large deletions were observed in 5.8% and 1.2%, respectively, and small indels were frequently detected in 59.3% (Supplementary Fig. 5d). These results indicate that PA provides more accurate integration outcomes than DSB-dependent HMEJ. This content has been added to the revised manuscript [Lines 197–202] and to Supplementary Figure 5d.

We also assessed genome-wide off-target integration events using Illumina short-read sequencing. Off-target integration was rarely detected in the HMEJ analysis, which is likely due to the low off-target activity of the sgRNA used in this experiment, predicted by Cas-OFFinder [PMID: 24463181]. In contrast, several off-target integration sites were identified in the eePASSIGE analysis, with the most frequently occurring off-target site accounting for 4.0% of the total sequence reads (Supplementary Fig. 5e). This content has been added to the revised manuscript [Lines 202–207] and to Supplementary Figure 5e.

Added Supplementary Figure 5d and 5e

2. Although PA was tested in patient-derived fibroblasts and primary human T cells, the reported editing efficiencies remain critically low, averaging only 1.9% (SF-PA), 2.4% (SFn-PA), and 1.3% (DF-PA) in fibroblasts, and 1.4% (SF-PA), 2.5% (SFn-PA), and 2.3% (DF-PA) in T cells. In contrast, eePASSIGE, another prime editing-derived method, has been reported to achieve 15–30% integration in primary human fibroblasts (https://doi.org/10.1038/s41551-024-01227-1) and over 60% integration of a 3.5-kb CAR cassette at the TRAC locus in T cells (https://doi.org/10.1182/blood-2023-181869). To properly position PA within the current technological landscape, a direct side-by-side comparison of PA and eePASSIGE under identical experimental conditions in these primary cell types is necessary. Functional assays of PA-engineered CAR T cells should also be included.

Response: Thank you for the comments.

i) We agree that the low PA-mediated replacement efficiency observed in patient-derived fibroblasts in the previous version was insufficient to support strong clinical applicability in that cell type. During this revision process, we found that the condition of patient-derived fibroblast lines was not good, compared to that of healthy individuals. Therefore, we decided to remove this data in the revised manuscript.

ii) Instead, during this revision process, we have focused on improving primary human T-cell experiments. In fact, our research team specializes in gene editing, but we have never worked with primary T cells before. This is the reason we obtained low editing efficiencies (1~3%) in primary T cells during the first revision process.

However, during this revision process, we sought to optimize PA treatment in T cell experiments. In particular, we found that the low survival rate of DNA-introduced T cells was likely a major bottleneck. We therefore improved cell recovery by optimizing the electroporation condition using B1mix buffer [PMID: 37500747] and modifying the post-electroporation culture conditions. As a result, CAR integration efficiencies were significantly improved across all PA systems, reaching 8.3% with SF-PA, 19.1% with SFn-PA, and 28.1% with DF-PA. Importantly, we confirmed that, under these conditions, electroporated T cells showed viability, apoptosis levels, and proliferation rates similar to control T cells electroporated with PE7 mRNA alone, and increased about 20-fold within 7 days to proliferate to more than 10^7 cells. We have added these results to revised Figure 5a-5e and description in the revised manuscript [Lines 298–316].

Updated Figure 5a-5c.

iii) To address the reviewer's concern, we also performed a direct side-by-side comparison between PA and eePASSIGE under matched target and delivery conditions in primary human T cells. Under the identical conditions, PA achieved 25.2% CAR integration efficiency, whereas eePASSIGE achieved 3.2%. In addition, we found that PA-engineered CAR-T cells exhibited antigen-dependent tumor cell killing and cytokine production, demonstrating that PA-mediated CAR integration can generate functional CAR-T cells.

However, as explained above, since CAR integration efficiency has significantly improved across all PA systems—reaching 8.3% in SF-PA, 19.1% in SFn-PA, and 28.1% in DF-PA—we believe that comparative data between PA and eePASSIGE does not need to be included in the revised manuscript. Therefore, it is provided for reviewers only, as follows.
Figure for the reviewer only.

iv) To further evaluate the in vivo applicability of PA, we prepared four plasmids containing PE7, two pairs of pegRNAs, and GFP donor, and all plasmids were subsequently delivered to mice via hydrodynamic tail-vein injection. Seven days after injection into mice, liver tissue was sorted by GFP fluorescence, and the average insertion efficiencies of 1.1% and 4.3% for DF-PA and SFn-PA, respectively. We have added these results to revised Figure 5f and Supplementary Figure 9 in the revised manuscript [Lines 317–327].

Newly added Supplementary Figure 9

Minor point 1. While the authors demonstrate PA's efficacy at clinically relevant loci (e.g., HTT, ALB, SLC39A14) in cell lines, the study lacks a compelling application scenario. Testing PA in a more physiologically relevant model or a specific therapeutic context is necessary to

convincingly demonstrate its utility and clinical translation potential.

Response: Thank you for the comment. As explained above, to more clearly demonstrate the practical utility of the PA method, we have improved the PA efficiency in human primary T cells from the existing 2% level to up to 28% through protocol optimization. Additionally, we performed new PA experiments in vivo using mice and obtained PA editing activity in mouse liver.

Minor point 2. Oligo sequences: The sequences of all guide RNAs and donor DNAs used in this study must be provided, preferably in the main text or as supplementary information.

Response: Thank you for the comment. In the revised manuscript, we have added the sequence information of all guide RNAs, pegRNAs, nicking guide RNAs, primers, probes, and donor-related oligonucleotides used in this study to the Supplementary Table 1-4.