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Reply to "Efficient Nuclease-free HR by Clade F AAV Requires High MOIs with High Quality Vectors"

We welcome Dr. Chatterjee's efforts to provide an explanation for the inability of our work in Rogers et al.,[1] as well as a similar study by Dudek and Porteus,[2] to reproduce the findings reported in Smith et al.[3] that clade F adeno-associated virus (AAV) vectors can promote homology-directed genome editing at high efficiency. Unfortunately, there are a number of mischaracterizations in her response that we would like to clarify.

Chatterjee argues that the studies in Rogers et al.[1] were not an appropriate replication of their work. The major concerns expressed were that we (1) only used homology donor constructs containing heterologous promoters; (2) did not perform appropriate molecular characterizations to confirm genome editing; (3) selected hematopoietic stem cell-derived AAV (AAVHSC) capsid sequences that were inappropriate variants to select and whose published sequences[4] differed from the "naturally occurring" material that they used; (4) used lower MOIs than were necessary to achieve genome editing; and (5) observed toxicity in $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) at higher MOIs and did not use appropriate culture conditions.

The first point is at odds with the fact that a significant portion of the data presented in Rogers et al.[1] (Figures 3–5) was generated using a promoter-less donor construct. Indeed, this AAVS1-targeted construct was designed specifically to match the reagents used in Smith et al.[3] and therefore to be an appropriate test of their system. The second point disregards our prior experience characterizing genome editing outcomes using AAV homology donors, which includes the use of deep sequencing and specific in-out PCR assays to confirm the insertion of larger gene cassettes.[5] As part of these analyses, any PCR amplicons so generated are validated by sequencing to confirm that they do indeed reflect precise gene insertion events.

Chatterjee also expressed concerns about the choice and origin of the AAVHSC capsids that we used to evaluate the findings in Smith et al.[3] Specifically, we generated recombinant AAV vectors containing capsids from the prototype clade F vector AAV9 (GenBank: AY530579.1) as well as the reported capsid sequence of AAVHSC13 (Smith et al.[4]). We are surprised by her dismissal of these capsids as not being an appropriate test of their data since both capsids were reported to support genome editing in K562 cells and $CD34^+$ HSPCs in Figure S1 of Smith et al.[3] A more extensive set of the AAVHSC variants described in their manuscript were similarly evaluated by Dudek and Porteus.

We are also puzzled by Chatterjee's dismissal of the clade F capsids we used as being "mutagenized capsid variants of AAV9" that differed from the "naturally occurring Clade F AAVs" that they used in their work. AAV9 is itself a well-characterized naturally occurring clade F serotype. For capsid AAVHSC13, we generated the capsid based on the sequence reported in Table 2 of the earlier paper from Chatterjee's group,[4] which indicates that the AAVHSC13 capsid is identical to AAV9 but with a G505R point mutation. This approach to recreating the AAVHSC capsids was also used by Dudek and Porteus.[2] In addition, and in contrast to Chatterjee's assertion, we did not claim that AAVHSC13 was identical to AAVHSC17. Indeed, we noted in our discussion that, although these two capsids have the same amino acid sequence, AAVHSC17 also contains an additional silent mutation in VP1 (Smith et al.[4]). We were unable to formally assess any contribution of this silent mutation because the specific DNA sequence was not reported.

The dismissal of our experimental system is further surprising to us given that Smith et al.,[3] in their discussion, attributed the unique behavior of the clade F vectors as arising from features of both the AAV genome structure and the clade F capsid sequences. Although a detailed description of the capsid plasmids used in Smith et al.[3] was not provided, the earlier paper from the group described the use of hybrid AAV2 *rep* plus AAVHSC *cap* constructs to generate AAVHSC vectors.[4] This approach is the most common method for generating recombinant AAV vectors,[6] which we also adopted. Similarly, the pSaiLuc vector genome backbone reported in Smith et al.[3] is based on AAV2, as is also standard practice, and we also used AAV2 vector genomes. If the work in Smith et al.[3] used a materially

different approach to generating recombinant AAV vectors, we look forward to that clarification.

Chatterjee also raises the possibility that our inability to achieve nuclease-independent genome editing was because we were not using clade F AAV vectors at sufficiently high MOIs, specifically at MOIs greater than 150,000. However, this statement is contradicted by their own data in Figure 1B of Smith et al.,[3] which shows that gene insertion rates between 5% and 15% were achieved in $CD34^+$ HSPCs when using much lower MOIs, in the range of 10,000 to 75,000. It also ignores the fact that we performed experiments to test whether MOI was a limiting factor, using MOIs of up to 500,000, in both HSPCs and 293T cells.

Of importance, as we discussed in Rogers et al.,[1] AAV titers are well-known to be difficult to compare between groups,[7,8] so direct comparisons of the stated MOIs between the different studies may only be of limited utility. Therefore, instead of focusing on a reported MOI, we consider it to be more useful to compare experiments that have been normalized based on the efficiency of AAV transduction. Toward that goal, Figure S2B of Smith et al.[3] shows transduction rates of 10% to 40% achieved by clade F vectors, which correlated with similar rates of genome editing. In contrast, although we were also able to transduce K562 and 293T cell lines with clade F vectors at rates of 5% to 50%, those same MOIs produced no genome editing events unless we also provided a targeted nuclease to generate a matched DNA break.

We concur with Chatterjee's comment that the toxicity we observed at an MOI of 500,000 in HSPCs impacted our ability to assess genome editing activity in these cells when using these high MOIs—a point that we acknowledged in our discussion. We also agree that this toxicity could occur as a result of some component of the vector preps and that different groups can certainly have qualitatively different preps. We further note that high MOIs and high rates of AAV transduction can themselves cause toxicity secondary to sensing of viral genomes, including through p53 activation.[9,10] Unfortunately, Smith et al.[3] provided only limited information about their own experience of toxicity in HSPCs: Table S2 provided data pooled from cells treated with different MOIs, and the range only spanned 10,000–150,000. In addition, as discussed above, absolute MOI values may not be directly comparable between our two groups.

New information is provided in Chatterjee's response that culture conditions and the cell cycle may be an important consideration for achieving nuclease-independent genome editing by clade F AAVs in HSPCs, and specifically that AAV transduction and genome editing are most efficient in slowly dividing HSPCs. This distinction, which was not noted in Smith et al.,[3] runs counter to reports that AAV transduction is enhanced in cycling cells due to increased second strand synthesis during S phase.[11] Moreover, this point seems to be at odds with data presented in Smith et al.[3] that genome editing by clade F AAVs also occurs in cell lines that are constantly dividing or that these genome editing events require BRCA2, which is limited to the S/G2 phases of the cell cycle.

In summary, although we appreciate Dr. Chatterjee's efforts to suggest an explanation for the discrepancies in our results, her comments do not yet explain the inability of our groups to reproduce the findings in Smith et al.[3] using recombinant AAV vectors containing clade F capsids. We continue to acknowledge that this may be due to some unappreciated or unreported aspect of the methods used and look forward to ongoing efforts toward reproducibility.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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https://doi.org/10.1016/j.ymthe.2019.11.003

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