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Translation Detection and Quantification Workflow to Characterize CRISPR-Cas Multiplex Edited Hematopoietic Stem and Progenitor Cells (HSPCs)

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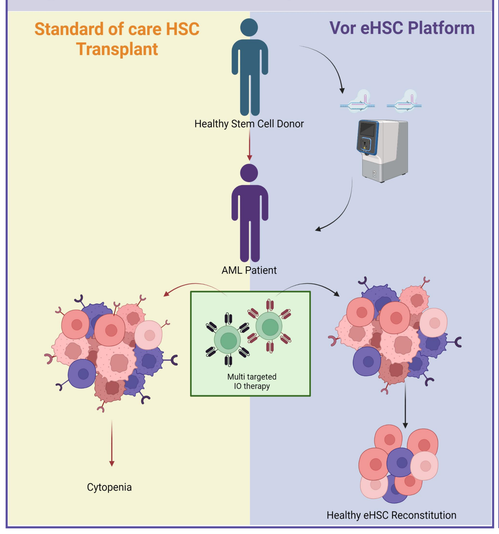
INTRODUCTION

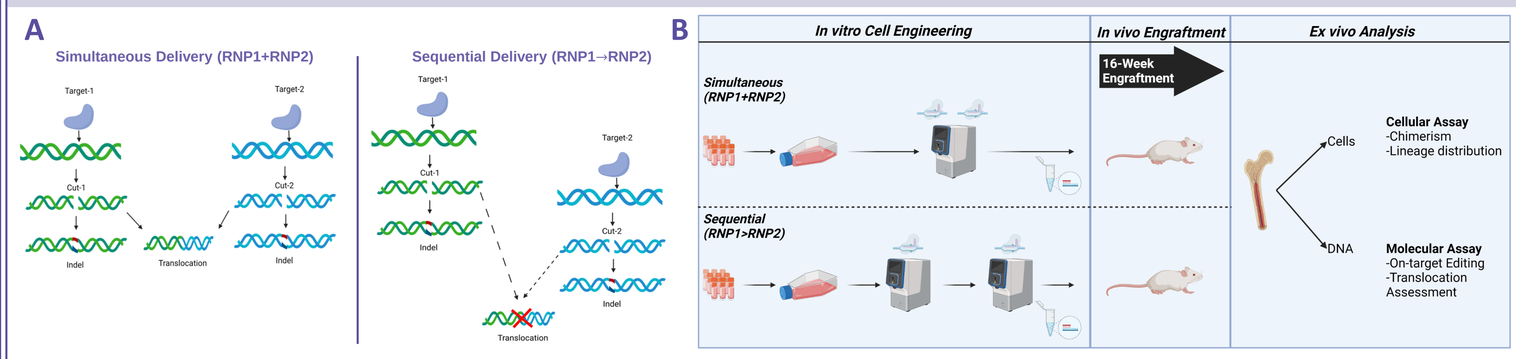
- Targeted immunotherapy of Acute Myeloid Leukemia (AML) has been limited due to lack of tumor-specific antigens resulting in “on-target, off-tumor” effects that can lead to severe cytopenia.
- To unlock the full potential of targeted treatments, we created treatment-resistant HSPCs by genetically ablating target antigens from healthy, HLA-matched donor-derived HSPCs for hematopoietic stem cell transplant.
- This allows compatible immuno-therapy to specifically kill leukemic cells bearing the AML target-antigen while protecting the target antigen-null allogeneic graft.
- Targeting multiple antigens simultaneously increases the potential to avoid antigen escape and addresses the issue of antigen heterogeneity of tumor cells.
- Multiplex engineering of HSPCs poses a risk of genomic translocations; here we describe a translocation detection and quantification workflow to fully characterize multi-edited cells.

Fig. 1. Vor eHSC Multiplex Platform



GENOME ENGINEERING METHODS

Fig. 2. Multiplex Delivery Strategies and Experimental Overview



- (A) Multiplex genome editing of HSPCs using CRISPR-Cas9 enables next generation transplant-based cell therapies but poses a risk of genomic translocation. Through optimized engineering approaches, that risk can be reduced. Here we compare a Simultaneous (Si) delivery to a Sequential (Se) delivery approach. Simultaneous delivery of two independent Cas9 RNP complexes leads to double stranded DNA breaks occurring in parallel resulting in an increased risk of translocation. Alternatively, multiplex engineering cells by applying a sequential delivery of two Cas9 RNP complexes allows time for the primary RNP to cut and repair before editing with the second RNP, which may lead to a reduction in translocation frequency.
- (B) To compare a Si and Se delivery of Cas9 RNP into HSPCs, CD34+ cells are thawed and allowed to recover in culture. Cell are then engineered in either a Si electroporation (EP) of 2 independent SpCas9 RNPs or a sequential (Se) EP where RNP1 is delivered, cells are recovered in culture, and then RNP2 is delivered in the second EP. After further culture, cell are harvested, and genomic DNA is extracted as input material is formulated for mouse dosing in preparation for long-term engraftment studies. After 16 weeks, bone marrow isolated and analyzed using cellular and molecular assays.

METHODS

Fig. 3. Bioinformatics Pipeline

- In-house developed bioinformatic pipeline analysis of each orthogonal assay informs the next in terms of identification and quantification of translocations species.
- The workflow represents the assay and data collection (green) and the bioinformatic analysis (blue).
- Design for each assay can be manipulated to explore on-target editing and translocation frequency.
- Identification of unknown species through unidirectional sequencing enables the design of targeted assays to resolve all translocation species.

Fig. 4. Sequencing Assay Design Enables Translocation Species Detection

- (A) Targeted NGS assays are designed to have specific primers to amplify and quantify on-target editing. With two multi-specific NGS panels, one acentric, one dicentric and two balanced translocation species (outlined in (C)) can be validated and quantified.
- (B) Unidirectional sequencing is based on a single primer that is designed on both sides of each cut site. This approach is a discovery tool that allows for the detection of all known/known translocation (all species in (C)). Furthermore, this system can also be used to detect known to unknown translocation events.

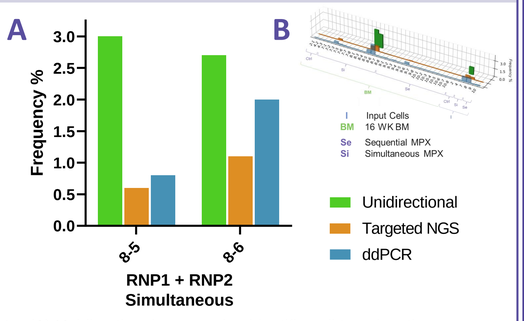
RESULTS

Fig. 5. On-Target Multiplex Editing Persists *in vivo*

- Representative on-target editing for all multiplex edited groups and results are consistent for both input material and 16-week bone marrow.
- Quantification of on-target editing by NGS at both target sites (RNP1 and RNP2) revealed no reduction in total editing when comparing the input material to the 16-week *ex vivo* bone marrow.
- The gene modifications in multiplex engineered cells persist through long-term engraftment studies.

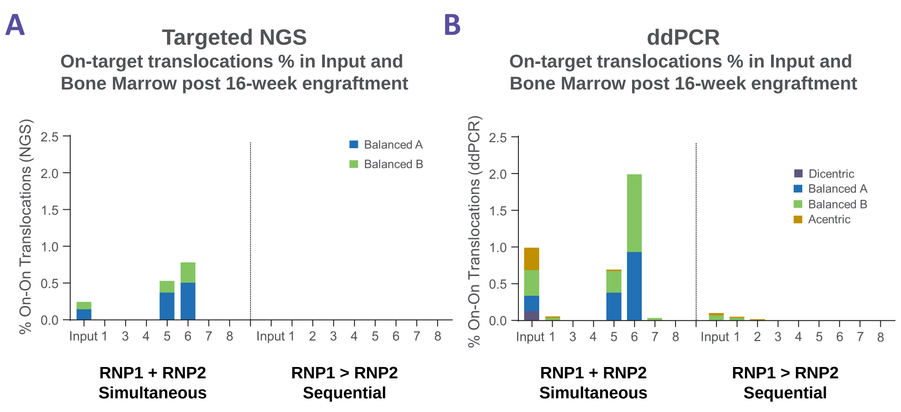
RESULTS

Fig. 6. Translocation Detection Methods



- (A) Unidirectional sequencing identifies all translocations including both on/on and on/off target translocations. Targeted NGS resolves 4 species of on/on target translocations with 2 NGS assays: acentric/dicentric and balanced/balanced. Finally, ddPCR quantifies the same 4 species of on/on target translocations as targeted NGS but with a higher degree of sensitivity. In this data set, only select samples were analyzed with unidirectional sequencing
- (B) Total translocations can be compared in both *in vitro* and *ex vivo* samples across all 3 assays to evaluate persistence of translocations.

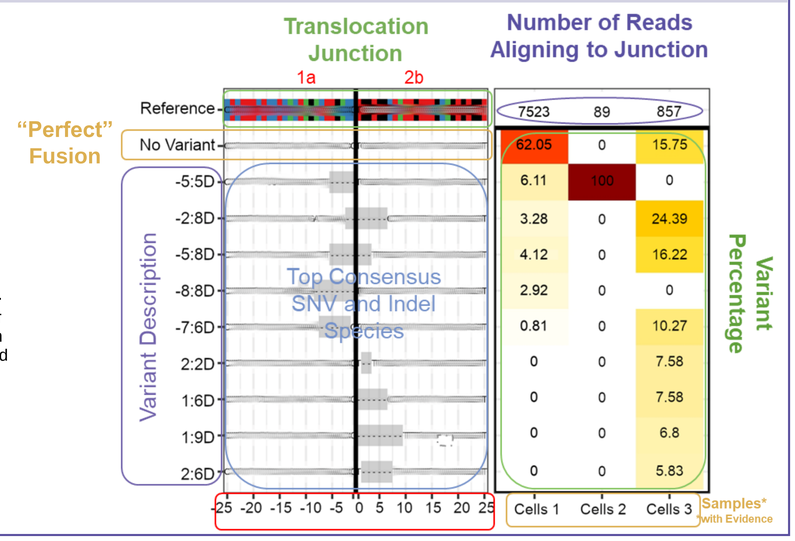
Fig. 7. Sequential Delivery Reduces Translocation Risk



- (A) Targeted NGS assays show a reduction in translocation frequency in both input material and *ex vivo* bone marrow post 16-week engraftment.
- (B) In parallel assays, ddPCR has an increased sensitivity which quantifies absolute frequency of specific translocation events. ddPCR confirms the overall reduction in translocation events in both input material and *ex vivo* bone marrow post 16-week engraftment.

Fig. 8. Translocation Species Informs Consequence

- NGS data is subjected to translocation consensus variant analysis. All translocation events are aligned to a translocation junction reference sequence constructed based on the predicted cut sites of each RNP target.
- Each translocation species then can be used as a reference to analyze the translocation events at base pair resolution to identify the exact translocation sequence.
- The total number of reads, for each sample, aligning to each translocation junction are used to report variant percentage.
- This data can be used to evaluate the potential consequence for all translocation events and informs the cell engineering approaches to reduce translocation risk.



CONCLUSION

- Multiplex genome engineered HSPCs have the ability to engraft and maintain persistence of editing in long-term mouse studies.
- Sequential multiplex delivery drastically reduces translocation events, as compared to a simultaneous delivery, in both input material and post long-term engraftment studies in a xeno-transplant mouse model.
- Our in-house developed bioinformatics pipeline can detect translocations in both unidirectional and targeted amplicon next generation sequencing data.
- We confirm detection of translocation using orthogonal approaches that are highly effective for resolving low abundance events.
- Full characterization of translocations and the resulting impact on cell biology enables design of multiplex editing strategies to produce safe and efficacious multi-gene modified cell products.
- Multiplex engineering of HSPCs enables the prospect of next-generation cell therapies for AML patients.

References

1. Clement K, Rees H, Canver MC, et al. CRISPResso2 provides accurate and rapid genome editing sequence analysis. Nature Biotechnology. 2019 Mar;37(3):224-226.

Disclosures

All authors listed above are current or former employees of Vor Biopharma

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