Maximizing safety and efficacy in hematopoietic stem cell manufacturing for gene therapy

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Introduction

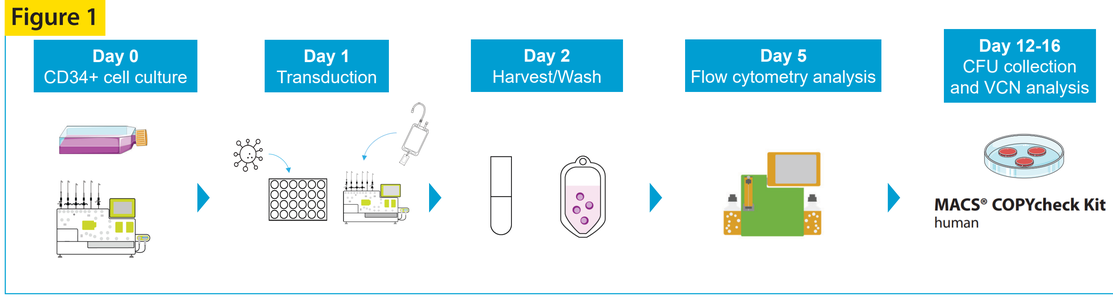
Hematopoietic stem cell (HSC) gene therapy has been recently approved for beta-thalassemia, sickle cell disease and adrenal leukodystrophy, specifically Zynteglo™, Lyfgenia™, Casgevy® and Skysona™. However, in the case of sickle cell disease, the challenges of high cost and scalability, as well as increased safety concerns regarding therapy-associated leukemic events indicate a need for improved and standardized manufacturing steps that allow for large scale implementation of gene therapy interventions. Automation is the key to achieve these goals and, to this end, the CliniMACS Prodigy® as an automated, closed, GMP-compliant platform for HSC transduction, is an ideal candidate to streamline cell processing. This device had previously demonstrated promising results in terms of cell viability, transduction efficiency and vector copy number (VCN) for HSC transduction, when a GFP encoding lentiviral vector[1] was utilized. In the current study, we intend to provide further insights on the performance of the CliniMACS Prodigy for HSC transduction by employing a transmembrane protein-encoding lentiviral vector, followed by assessment of the transduction efficiency, CFU analysis, VCN determination and *in vivo* engraftment studies.

Methods

1 Workflow for manual and automated lentiviral transduction

Following CD34+ enrichment from human mobilized leukapheresis, CD34+ cells are cultivated for one day either in open steps (manually) or on the CliniMACS Prodigy (Hematopoietic Stem Cell Engineering software) on the tubing set 520. On day 1, CD34+ cells are transduced with the VSVg pseudotyped CD19 CAR lentiviral vector (LV) at MOI 30. On day 2, gene engineered CD34+ cells are harvested from the CliniMACS Prodigy or washed in the manual process and they are then analyzed by flow cytometry at day 5 to assess the transduction efficiency. Before and after transduction, CFU assays are performed and will be counted and characterized at day 12-16. Additionally, we collected individual CFUs to carry out the VCN analysis and flow cytometry analysis to assess the transduction efficiency at CFU level (Fig.1).

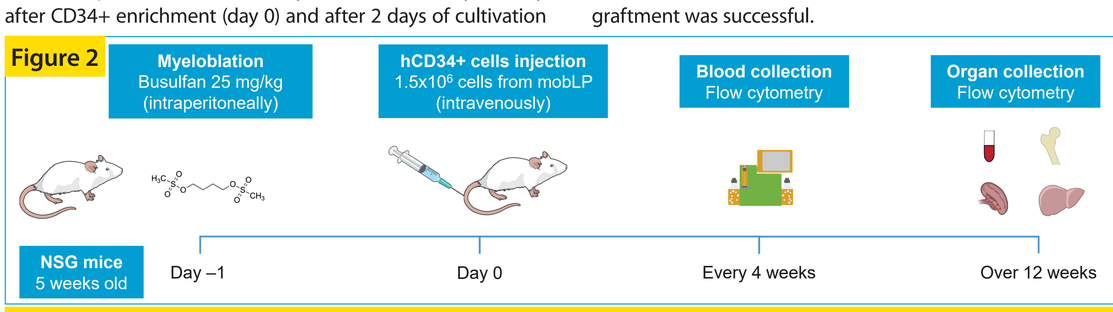
Figure 1



2 *In vivo* experimental design

5 weeks old NSG mice received intraperitoneal injection of 25 mg/Kg of Busulfan as myeloablative conditioning regimen. After 24 hours, 1.5x10^6 human CD34+ cells derived from mobilized leukapheresis (mobLP) are injected intravenously, directly after CD34+ enrichment (day 0) and after 2 days of cultivation (transduced and untransduced conditions). Every 4 weeks blood is collected to investigate the level of humanization and after 12 to 16 weeks the mice are euthanized and blood, spleen, liver and bone marrow are collected to evaluate whether the engraftment was successful.

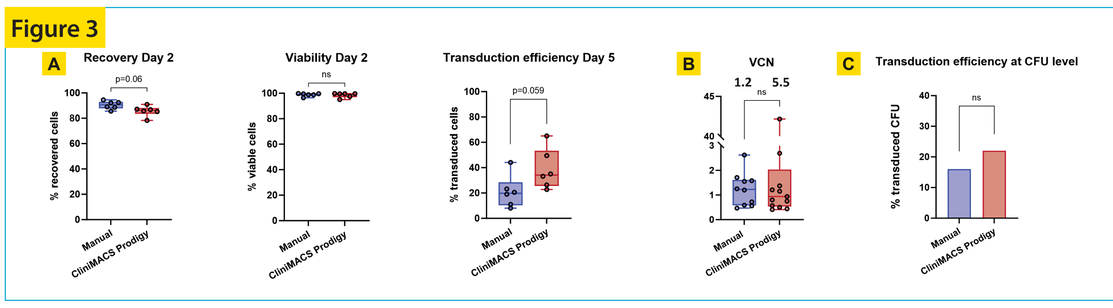
Figure 2



Results

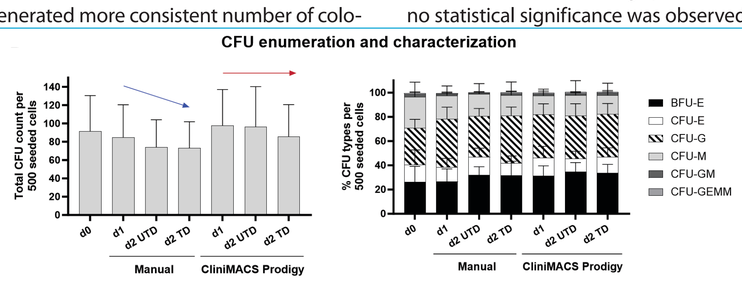
CD34+ cells were transduced in parallel in open steps (manually) and on the CliniMACS Prodigy (automated) with a VSVg CD19 CAR LV without the addition of any transduction enhancers. The results showed comparable cell recovery and viability between the manual and automated workflows, but superior performance of the automated platform in terms of transduction efficiency (Fig.3A). For the calculation of VCN, individual CFUs were picked and analyzed via qPCR using the MACS® COPYcheck Kit. As expected, there was an increase of the VCN for the CliniMACS Prodigy (average 5.5) compared to the manual steps (average 1.2), consistent with the higher transduction efficiency observed (Fig.3B). Analyzing the % of transduced colonies and calculating the transduction efficiency at CFU level, it shows an overall decrease compared to the transduction efficiency evaluated by flow cytometry at day 5, but still maintaining a higher value in the automated setting (Fig. 3B).

Figure 3



In order to investigate the effect of cultivation and transduction on the HSC clonogenic capacity, we tested the cells' potential to differentiate into various hematopoietic lineages on day 12-16 post-transduction by standard colony forming unit (CFU) assay. The results showed fluctuations in the CFU count among the conditions, however the transductions performed on the CliniMACS Prodigy generated more consistent number of colonies compared to the counts of CFUs generated when the HSCs were cultivated under manual conditions. Moreover, we noticed a clear trend of decreased number of CFUs from the manual steps (see blue arrow in Fig.4), whereas the number of colonies deriving from the CliniMACS Prodigy remained almost stable (see red arrow in Fig.4). It should be noted however that no statistical significance was observed in these experiments.

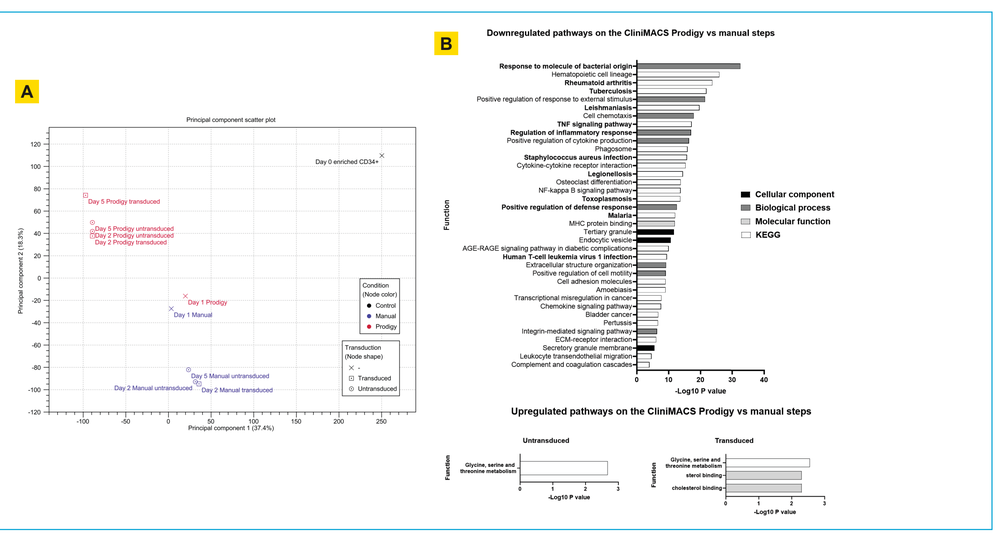
Figure 4



To provide a plausible explanation about the higher transduction efficiency and the more sustained clonogenic capacity of the automated process, we conducted bulk transcriptome profiling via next generation RNA sequencing (bulk RNAseq) across all conditions, comparing samples from manual and automated processes at several timepoints (D0, D1, D2 and D5). Results from the principal component analysis (PCA) highlighted that samples cultivated on the CliniMACS Prodigy cluster closer to the control sample at D0, indicating that the automated cultivation process allows the preservation of cellular features similar to the original condition at the experimental onset (Fig. 5A) and further corroborating the CFU analysis data. From the samples processed on the Prodigy (untransduced and transduced), we observed:
- downregulation of overall immune responses (in bold);
- downregulation of CD38 gene (suggesting higher stemness);
- downregulation of Toll-Like Receptor 8 (for ssRNA, suggesting the endosomal escape of the LV);
- upregulation of cholesterol/sterol binding pathways (with LDL-R gene upregulated on the day of transduction).

Overall, these differentially expressed gene pathways could explain the promotion of lentiviral transduction on the Prodigy. In addition, the samples transduced on the Prodigy showed upregulation of glycine, serine and threonine metabolism, suggesting enhanced cell fitness[2] (Fig.5B).

Figure 5



Our results so far indicated that HSCs maintain better their stem cell character when cultivated on the CliniMACS Prodigy, and therefore, the natural extension of the experimental approach would be to consider these results in the context of *in vivo* studies. Our results indicated:

- Engraftment of hCD45+ cells in the mouse bone marrow (mBM) is better preserved with cells processed on the Prodigy (Fig.6A);
- The % of engrafted hCD34+ cells decreased in transduced samples, but with lower variability on the Prodigy (Fig.6B);
- The % of GFP+/CD34+ cells showed comparable transduction efficiency to the data obtained by flow cytometry and comparable between the manual and automated methods (Fig.6C);
- No significant differences were detected in the % of hCD133+ and hCD117+ cells (long-term HSCs) among the conditions (Fig.6D-E).
- The gating strategy applied for flow cytometry analysis of the *in vivo* studies is shown in Figure 7.

Figure 6

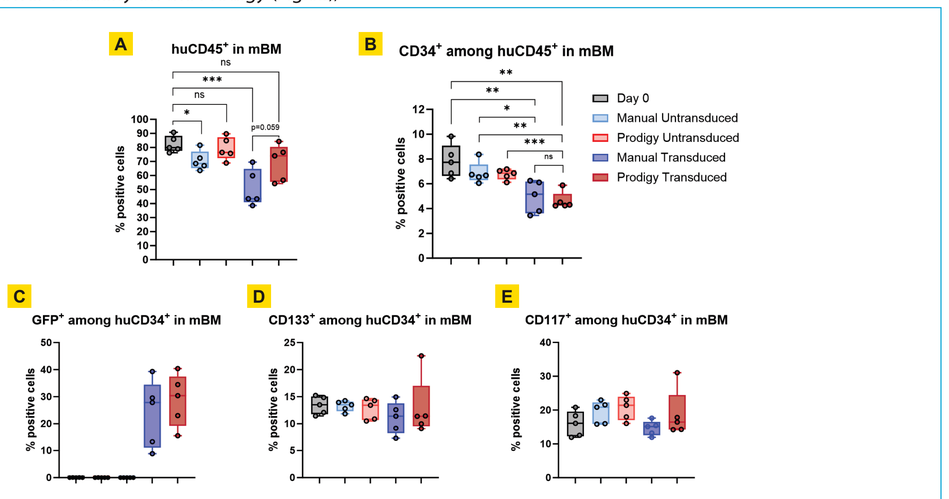
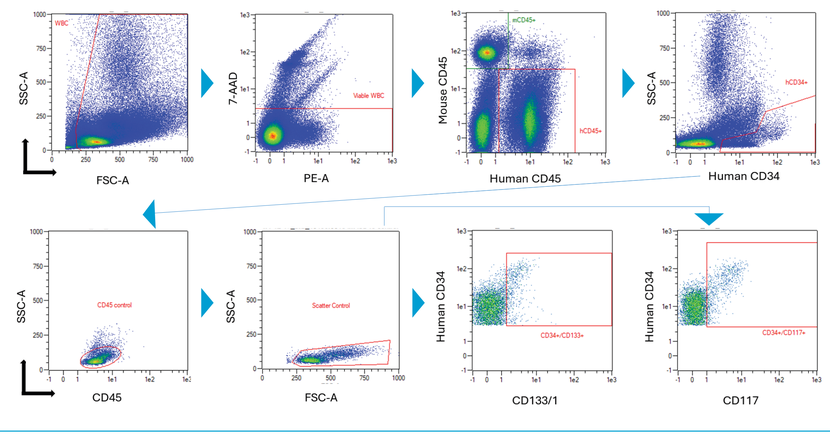


Figure 7



Conclusions

- Our results indicate that CD34+ cells transduced with a VSVg LVs present higher transduction efficiency on the CliniMACS Prodigy compared to manual steps, corroborating previous results[1];
- There is a trend towards better fitness of HSCs cultivated on the CliniMACS Prodigy as evidenced by:
  a) the constant number of colonies generated in CFU assays;
  b) the bulk RNAseq analysis showing upregulation of the glycine, serine and threonine metabolism;
  c) the results from the PCA analysis demonstrating that CD34+ cells cultivated on the Prodigy are closer in terms of gene expression profiles to CD34+ cells from day 0 (baseline);
  d) the better huCD45+ cells engraftment compared to cells processed manually considering as gold standard the unmanipulated CD34+ cells directly after enrichment i.e. D0, suggesting that the Prodigy promotes at least better engraftment for short term HSCs;
- VCN is increased in the samples processed on the CliniMACS Prodigy, consistent with the higher transduction efficiency observed by flow cytometry and at the CFU level;
- The engraftment of huCD34+ cells decreased in transduced samples but with lower variability on the Prodigy.

References

1. Papanikolaou, E. *et al,* Blood 2019, 134; S1, 5753.
2. Aon, M.A. *et al,* Cell.Metab. 2020, 32; 1, 100-116.

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