High Yield and Purity Differentiation of Human Induced Pluripotent Stem Cells (iPSCs) to Hematopoietic Progenitor Cells (HPCs) in a Scalable Stirred Tank Bioreactor

Notch THERAPEUTICS

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OVERVIEW

Human induced pluripotent stem cells (iPSCs) are a highly consistent cell source for the ex-vivo production of hematopoietic progenitor cells (HPCs) due to their potential for unlimited expansion. HPCs in turn are starting material for the generation of CD8αβ+ T cells using Notch's proprietary differentiation technology. Conventional static culture protocols for iPSC differentiation lack the process control, intensity and scalability needed for clinical manufacturing of T-cell based cancer immunotherapy products. Stirred suspension-based bioprocessing offers scalability and control of the complex interplay of various processing parameters, yet has not been widely adopted for iPSC manufacturing due to the challenge of moving from static to agitated culture systems.

Our aim was to translate a static, well-plate HPC differentiation protocol to a stirred tank bioreactor (STR) to establish the scalability of the process. We subsequently verified the suitability of the bioreactor platform to produce lymphoid-competent HPCs for use in downstream T cell differentiation. To increase process efficiency of aggregate based cultures in STR, we implemented 1) optimization of agitation rate to achieve the desired aggregate formation and size, 2) kinetic evaluation of aggregate morphology to identify the time of harvest that results in high purity of HPCs, and 3) perfusion feeding to improve process efficiency and yield of HPCs.

Highlights:

- Hematopoietic progenitor cells (HPCs) can be generated from induced pluripotent stem cells (iPSCs) in a scalable protocol with high yield, viability, and purity.
- Increased process efficiency of aggregate based cultures in STR via implementation of aggregate dissociation, perfusion feeding, and in-process monitoring.
- iPSC-derived HPCs are multipotent and lymphoid-competent, generating CD8αβ+ T cells in downstream differentiation.

METHODS

Stirred Tank Reactor (STR) culture

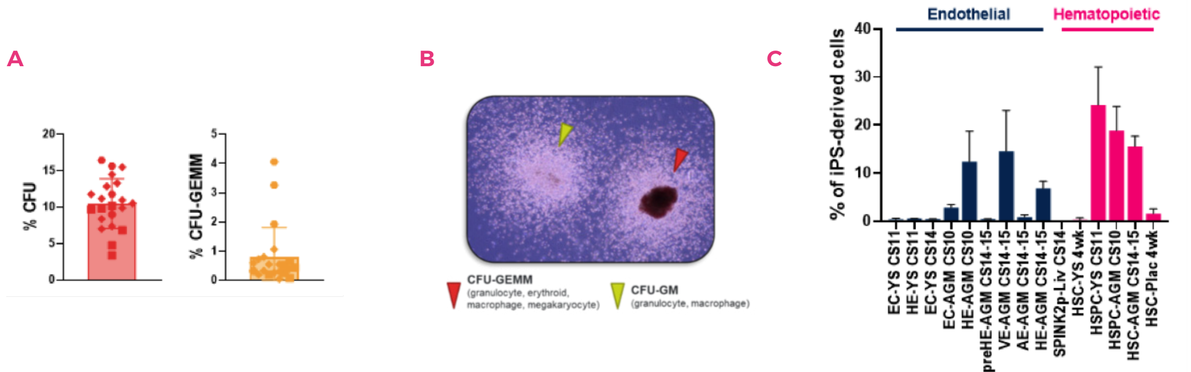
DASbox Mini Bioreactors (Eppendorf AG) were inoculated with pre-conditioned iPSC in a final culture volume of 140 mL. Impeller speed was set at low and high rpm to modulate the aggregate formation and diameter. Perfusion was established by incorporating a porous outflow filter as a cell retention device. Cells were cultured for 10 days to generate HPCs by applying the same flow rate for addition of feed and removal of depleted waste.

Analytics used for cell characterization

- Cell counts and viability
- Colony-Forming assays (CFU) and scRNAseq processing
- Flow cytometry-based phenotype of mesoderm and hematopoietic markers
- Aggregate sizing and number throughout differentiation
- Metabolic Profile

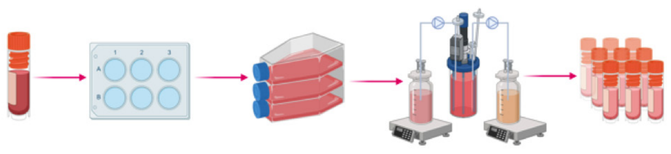
RESULTS

Figure 1. Research scale CD34 differentiation protocol was established, leading to efficient generation of iPSC derived CD34+ cells capable of generating multipotent GEMM colonies and resembling hematopoietic progenitors and endothelial cells from tissues in the early human embryo



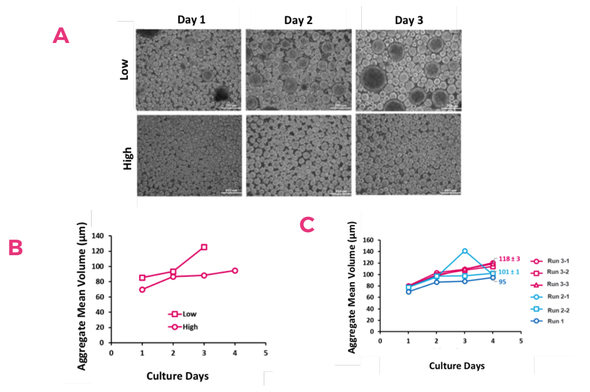
A In the CFU assay, 10.5 ± 0.7% of enriched CD34+ cells generated colonies, and 0.8 ± 0.2% of cells formed multipotent GEMM colonies. **B** Representative image showing CFU-GM and CFU-GEMM colonies. **C** iPSC derived CD34+ HPCs (n=3) were assessed by scRNAseq and compared to those from in vivo hematopoietic tissues in the human embryo described in Calvanese et al.[1] iPSC derived CD34+ cells most resembled endothelial and hematopoietic cell types found in Carnegie Stage (CS) 10-15 human embryos.

Figure 2. iPSC differentiation to HPCs in a stirred suspension bioreactor



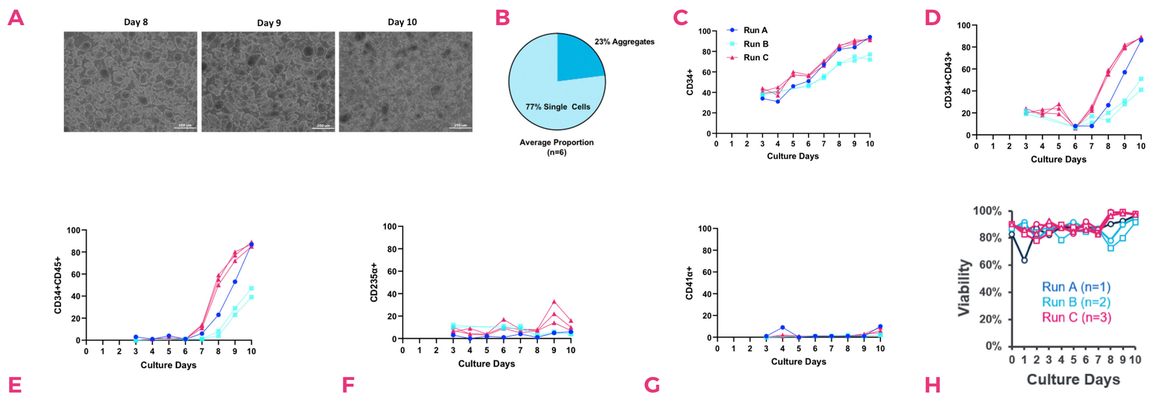
A schematic of iPSC differentiation to CD34+ cells. A cryovial of banked iPSCs is thawed and seeded onto a coated 6 well plate and expanded in static culture in T flasks. Cells are dissociated from monolayer cultures and seeded as single-cell suspensions at a defined seeding density into a stirred tank bioreactor (STR). Cells are allowed to form aggregates with continuous stirring and differentiated to CD34+ cells over 10 days using a combination of cytokines, growth factors and small molecules in a basal media.

Figure 3. Optimization of stirring rate enabled desired aggregate formation



A Stirring rate in the STR was optimized based on the research scale benchmark (in the ULA and spinner flask) of cell aggregates of ~70µm on day 1. Within the range of stirring rates tested, this target was achieved with the high stirring rate. Representative light microscopy images of low vs high stirring process derived aggregates on days 1, 2, and 3 (scale bar = 250 µm) shows high degree of aggregate heterogeneity in the bioreactor kept at low stirring speed. **B** Lower stirring rate significantly increased the aggregate size during differentiation (120 µm) whereas higher stirring enabled aggregate size control (80 µm) and overall size reduction. **C** All reactor vessels showed very similar aggregate sizing profile by number and %volume (95- 118 µm) at high stirring speed. (mean ± SEM, 3 independent STR runs, n=6 bioreactors).

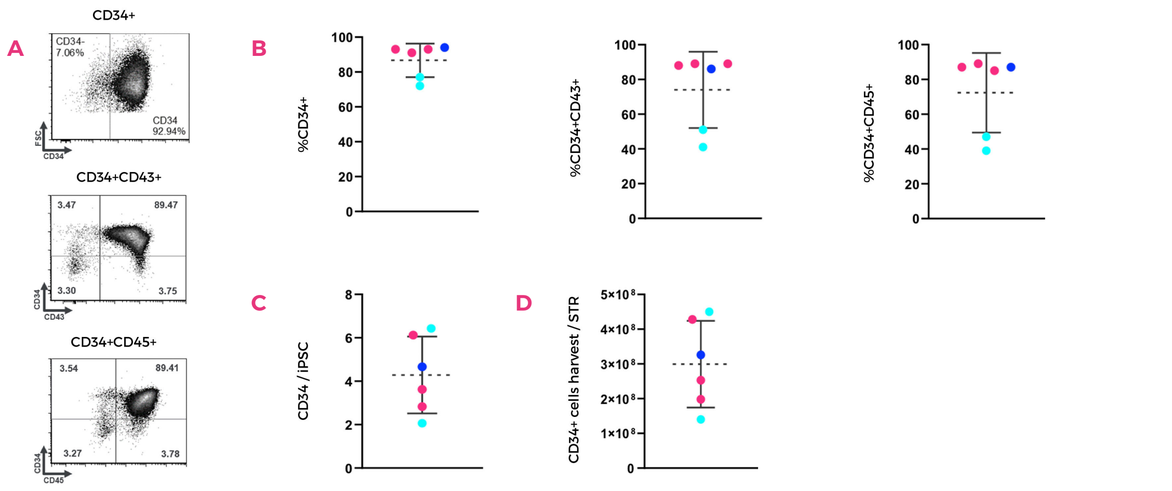
Figure 4. Kinetic evaluation of phenotype and morphology identified timing of harvest that achieved high purity HPCs



A Representative bursting morphology on day 8, 9 and 10. Earliest bursting phenotype was observed on day 8 with the extensive aggregate bursting on day 10 across all reactor vessels in 3 different runs (scale bar = 250 µm). **B** High degree of single cell fraction was observed (77 ± 10%) compared to aggregates (23 ± 10%) on day 10. Busting phenotype of aggregates to single cells was associated with the increased purity of HPCs fraction as seen with **C** CD34+, **D** CD34+CD43+, and **E** CD34+CD45+ cells. CD34+ expression increased from ~30% at day 3 to >75% at day 10. CD43+ and CD45+ expression was <10% by day-7 but extending the culture condition to day-10 significantly increased their expression across all three STR runs. Extending the culture harvest day to day 10 led to higher HPCs fractions without increase in the alternate lineages such as **F** erythroid (CD235α+) or **G** megakaryocyte (CD41α+). **H** High overall viability was maintained at levels >80% across all STR runs throughout the 10-day differentiation process (mean ± SEM, 3 independent STR runs, n=6 bioreactors).

RESULTS CONT'D

Figure 5. Optimized process conditions in STR generated HPCs with a high frequency of CD34+, CD43+ and CD45+ HPCs



A Flow cytometry plots showing the phenotype of a representative HPCs generated in STR vessel from iPSCs. Most of the CD34+ cells co-express CD43+ and CD45+ HPCs, and do not express CD235α or CD41α (not shown). **B** These cells had an average purity of 86.7 ± 3.9% CD34+ at harvest without enrichment. CD43+ HPC purity was 74.0 ± 8.9%, and CD45+ HPC purity was 72.0 ± 9.2%. **C** Approximate yield of CD34+ cells were 3.4 ± 0.8% per undifferentiated iPSC, and **D** total CD34+ cells harvested per STR were 3E8 ± 0.5 CD34+ cells per 140 mL vessel volume. (mean ± SEM, 3 independent STR runs, n=6 bioreactors).

Figure 6. Lymphoid potential of iPSC derived CD34+ cells

A Overview of Notch's proprietary 3D Engineered Thymic Niche (ETN) platform for scalable T-cell manufacturing using iPSC derived CD34+ HPCs. The Notch ETN is amenable for use in STR to differentiate CD34+ HPCs to CD8 single positive T cells. **B** iPSC derived CD34+ HPCs from 3 different STR runs were differentiated to CD8 cells using microplate culture with the research platform derived HPCs as a reference. STR derived HPCs were equivalent to research platform derived cells and generated 58.7 ± 3.7% CD8+ cells compared to 59.99% CD8 positive cells from the research platform HPCs. **C** The expression pattern for the CD8αβ+ T cells was equivalent across all 3 STR runs (mean ± SEM, 3 independent STR runs, n=6 bioreactors).

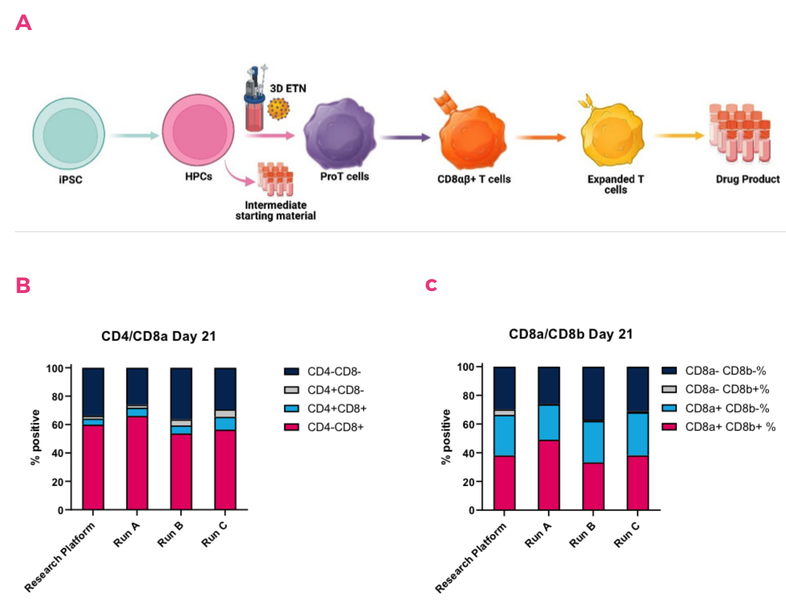
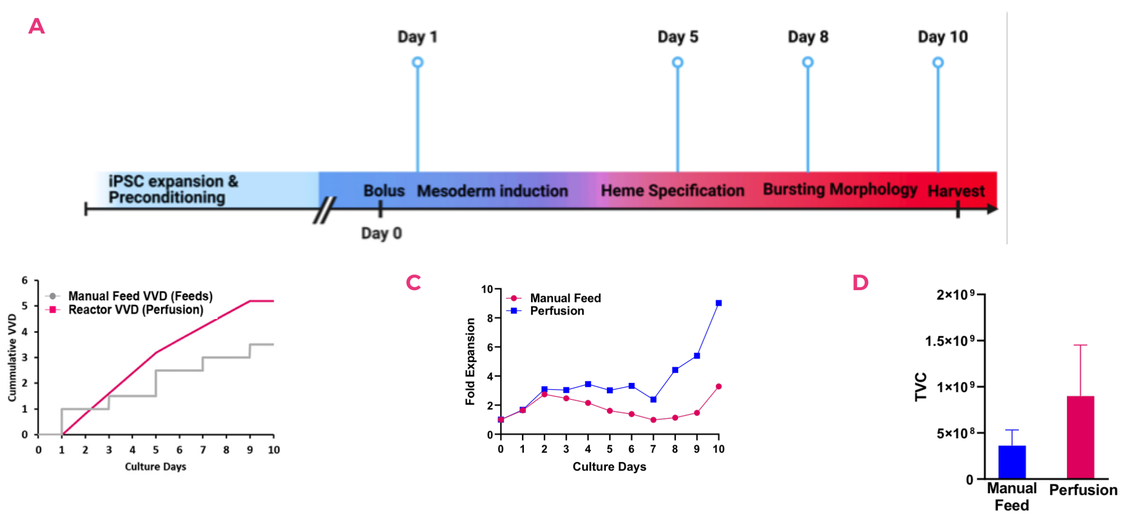
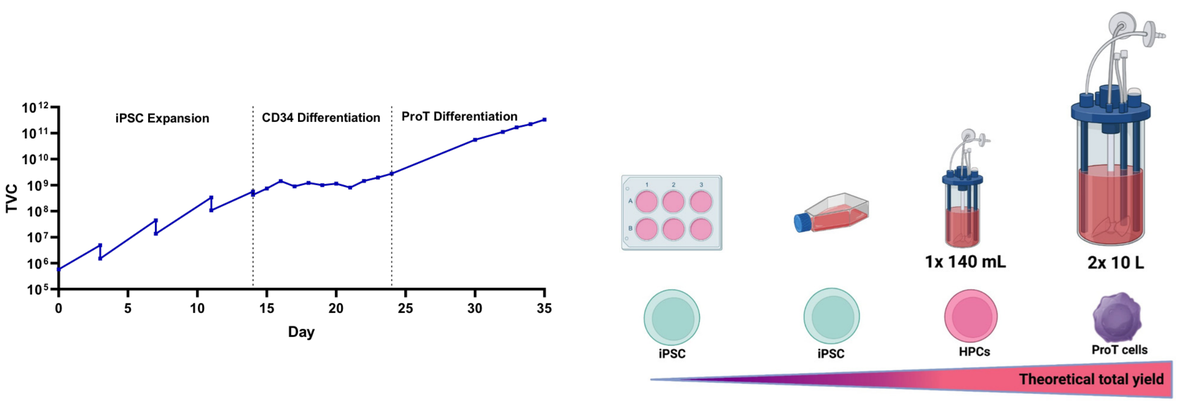


Figure 7. Implementation of perfusion feeding increased expansion of HPCs and highlights potential of further process development



A To avoid repeated feeding interventions required for a manual feeding condition and allow for a continuous medium exchange a perfusion feeding was started after 24 hours of culture initiation. **B** Rate of perfusion was set to reach a total of ~6.0 vessels volumes over a period of 9 days. In comparison, repeated fresh feeding is required with the manual feed intervention. **C** Perfusion feeding was demonstrated to achieve greater fold expansion throughout the differentiation culture. **D** Perfusion feeding enabled 2-fold increase in the total yield of HPCs allowing for ~1E9 HPCs from a single STR vessel with working volume 140 mL (mean ± SEM, n=3).

Figure 8. Scalable STR culture for manufacturing iPSC derived HPCs will enable small footprint manufacturing and support production of clinical and commercial scale T cells



A single small footprint 250 mL STR vessel (at 140 mL working volume) can generate the starting material to seed 2x 10L STR vessels for the next stage of T cell differentiation. Future scale up of the CD34 process to 1 L is projected to support clinical manufacturing.

CONCLUSIONS

- High yield and high purity HPCs can be manufactured from iPSCs in a well controlled and scalable STR system.
- The use of the STR platform will allow for further scale up of our culture processes and future clinical manufacturing for off-the-shelf allogeneic cell therapies.

References:

1. Calvanese, V. *et al.* Mapping human haematopoietic stem cells from haemogenic endothelium to birth. *Nature* 1–7 (2022) doi:10.1038/s41586-022-04571-x.